

Biochemical Pharmacology

Lecture Notes

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About these notes

These course notes have been assembled during several classes I taught on Biochemical Pharmacology. I welcome corrections and suggestions for improvement.

Chapter 1. Introduction

What is 'biochemical pharmacology'?

- A fancy way of saying 'pharmacology', and of hiding the fact that we are sneaking a subject of medical interest into the UW biochemistry curriculum.
- An indication that we are not going to discuss prescriptions for your grandmother's aching knee; we will focus on the scientific side of things but not on whether to take the small blue pill before or after the meal.

What is it not?

- A claim that we accurately understand the mechanism of action of each practically useful drug in biochemical terms.
- A claim that enzyme mechanisms and receptor structures, or even cell biology suffice as a basis to understand drug action in the human body (how do you measure blood pressure on a cell culture?). In fact, we are going to spend some time with physiological phenomena such as cell excitation and synaptic transmission that are targeted by many practically important drugs.

1.1. What are drugs?

Do drug molecules have anything in common at all? Figure 1.1a shows the structure of the smallest drug - molecular (or, more precisely, atomic) weight 6 Da.

On the other end of the scale, we have a rather large molecules - proteins. Shown is the structure of tissue plasminogen activator (t-PA; Figure 1.1b). t-PA is a human protein. Its tissue concentration is very low, but by means of recombinant expression in cell culture it can be obtained in clinically useful amounts. t-PA is now the 'gold standard' in the thrombolytic therapy of brain and myocardial infarctions.

The molecular weight of t-PA is about 70 kDa. Few drug molecules (among them the increasingly popular botulinum toxin) are bigger than t-PA.

More typical sizes of drug molecules are shown in Figure 1.2. Most practically useful drugs are organic molecules, with as molecular weight of roughly 200 to 2000, mostly below 1000. Interestingly, this also applies to many natural poisons (although on average they are probably somewhat larger). Are there reasons for this?

Reasons for an upper limit include:

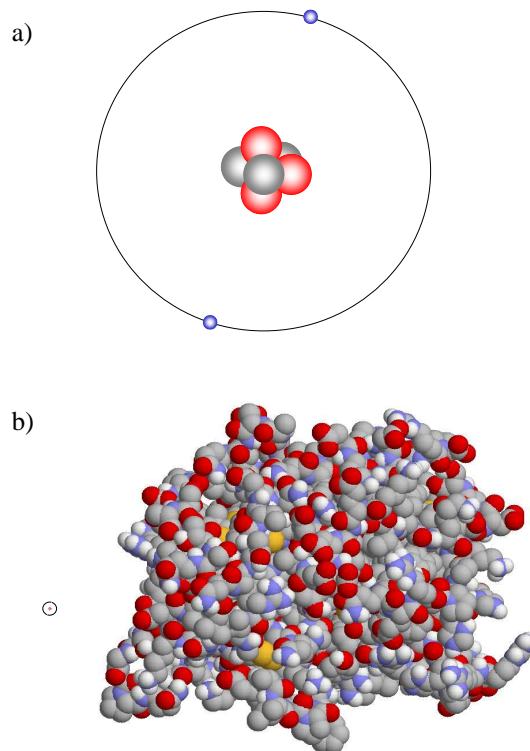


Figure 1.1. A small drug and a large one. a: Lithium is a practically very important drug in psychiatry. Its mode of action is still contentious - we will get into this later on in this course. b: Tissue plasminogen activator is a protein that is recombinantly isolated and used to dissolve blot clots. Lithium is shown on the left for comparison.

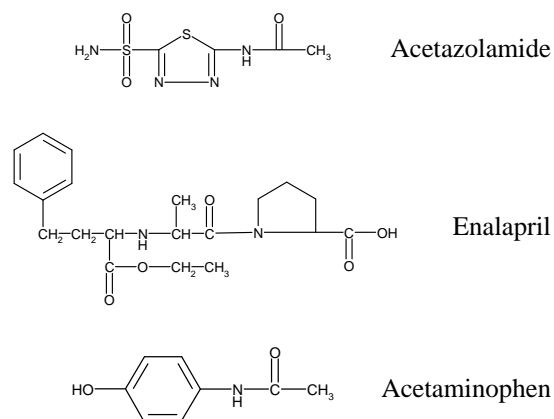


Figure 1.2. Some randomly chosen examples of drug molecules to illustrate typical molecular size. These drugs are all enzyme inhibitors but other than that have nothing in common. (Acetazolamide inhibits carbonic anhydrase, enalapril inhibits angiotensin converting enzyme, and acetaminophen inhibits cyclooxygenase.)

1. Most drugs are chemically synthesized (or at least modified, e.g. the penicillins) – the larger the molecules, the more difficult the synthesis, and the lower the yield will be.
2. Drugs need to reach their targets in the body, which means they need to be able to cross membrane barriers by diffusion. Diffusion becomes increasingly difficult with size.

One argument for a lower limit may be the specificity that is required – drugs need to act selectively on their target molecules in order to be clinically useful. There are numerous examples of low-molecular weight poisons – probably the better part of the periodic table is poisonous. There are, however, interesting exceptions to these molecular size rules of thumb. One is lithium; another popular example is shown in Figure 1.3.

1.2. Drugs and drug target molecules

Drugs need to bind to target molecules. Is there anything remarkable about this statement at all? Well, two things:

1. It is a surprisingly recent insight – only about 100 years old. (OK, so that is relative – long ago for you, but I'm nearly there.)
2. It is not generally true.

The idea of defined receptor molecules for drugs or poisons was conceived by Paul Ehrlich (Figure 1.4). Ehrlich worked on a variety of microbes and microbial toxins. He observed

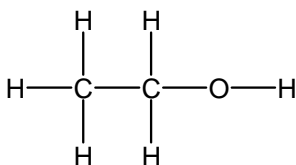


Figure 1.3. An interesting exception to the molecular size rules of thumb.



Figure 1.4. Paul Ehrlich. Paul Ehrlich was a German Jewish physician and scientist, who was inspired by and initially worked with Robert Koch (who discovered the causative bacterial agents of Anthrax, Tuberculosis, and Cholera). Left: Ehrlich's portrait on a 200 deutschmarks bill (now obsolete).

that many dyes used to stain specific structures in microbial cells in microscopic examinations also exerted toxic effects on the microbes. This observation inspired him to systematically try every new dye he could get hold of (and new dyes were a big thing in the late 19th century!) on his microbes. Although not trained as a chemist himself, he managed to synthesize the first effective antibacterial drug – an organic mercury compound dubbed 'Salvarsan' that was clinically used to treat syphilis for several decades, until penicillin became available. Ehrlich screened 605 other compounds before settling for Salvarsan. In keeping with his enthusiasm for colors and dyes, Ehrlich is credited with having possessed one of the most colorful lab coats of all times (he also had one of the most paper-jammed offices ever). His Nobel lecture (available on the web) is an interesting read – a mix of brilliant and utterly 'naive' ideas that makes it startlingly clear how very little was known in biology and medicine only a century ago.

So, what molecules *are* targets of drugs? Some typical examples are found in the human renin-angiotensin system, which is important in the regulation of blood pressure (Figure 1.5). Angiotensinogen is a plasma protein that, like most

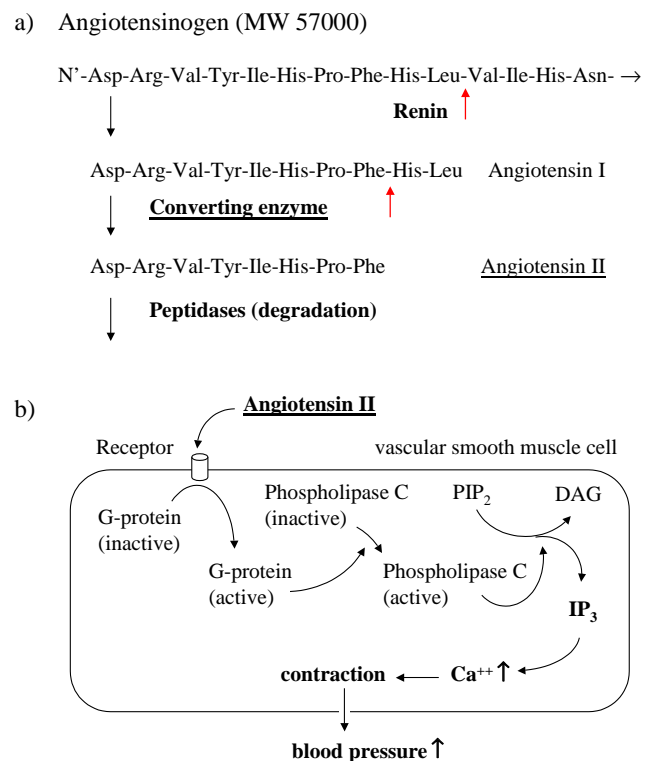


Figure 1.5. The renin-angiotensin system. a) Angiotensinogen is cleaved site-specifically by renin to yield angiotensin I. The latter is converted by another specific protease (angiotensin convertase or converting enzyme) to angiotensin II. b) Angiotensin effects vasoconstriction by acting on a G protein-coupled receptor that is found on smooth muscle cells. This ultimately leads to increased availability of free Ca⁺⁺ in the cytosol and contraction of the smooth muscle cells.

plasma proteins, is synthesized in the liver. From this protein, the peptide angiotensin I is cleaved by the specific protease renin, which is found in the kidneys (*ren* lt. = kidney). Angiotensin I, which is only weakly active as a mediator, is cleaved further by angiotensin converting enzyme, which is present in the plasma. This second cleavage releases angiotensin II, which is a very powerful vasoconstrictor. Angiotensin II acts on a G protein-coupled receptor, a membrane protein that is found on vascular smooth muscle cells. Through a cascade of intracellular events, this receptor triggers contraction of the muscle cell, which leads to constriction of the blood vessels and an increase of blood pressure).

Increased activity of the renin-angiotensin system is frequently observed in kidney disease, which may lead to abnormally high release of renin. Several points in the system are amenable to pharmacological inhibition. The first one is renin itself, which splits a specific bond in the angiotensinogen polypeptide chain (Figure 1.5a). An inhibitor of renin is remikiren (Figure 1.6a).

Remikiren (Figure 1.6a) is effective but has several shortcomings, such as low ‘bioavailability’ – which means that the drug does not efficiently get into the systemic circulation after oral uptake. Of course, oral application is quite essential in the treatment of long-term conditions such as hypertonia. A major cause of low bioavailability of drugs is their metabolic inactivation. Drug metabolism mostly happens in the liver (and sometimes in the intestine) and often is a major limiting factor of a drug’s clinical usefulness. Remikiren contains several peptide bonds, which likely are a target for enzymatic hydrolysis.

The most practically important drugs that reduce angiotensin activity are blockers not of renin but of angiotensin converting enzyme blockers, such as enalapril (Figure 1.6b). These have a major role in the treatment of hypertonia. In contrast to remikiren, enalapril is of smaller size and has only one peptide bond, which is also less accessible than those of remikiren. These features correlate with a bioavailability higher than that of remikiren.

1.3. Drug molecules may or may not have physiological counterparts

The vasoconstricting action of angiotensin can also be countered at the membrane receptor directly. One such inhibitor that has been around for quite a while is saralasin (Figure 1.6c).

Saralasin illustrates that the structure of the physiological mediator or substrate is a logical starting point for the synthesis of inhibitors. However, it is not a completely satisfactory drug, because it cannot be orally applied – can you see why? The more recently developed drug valsartan (Figure

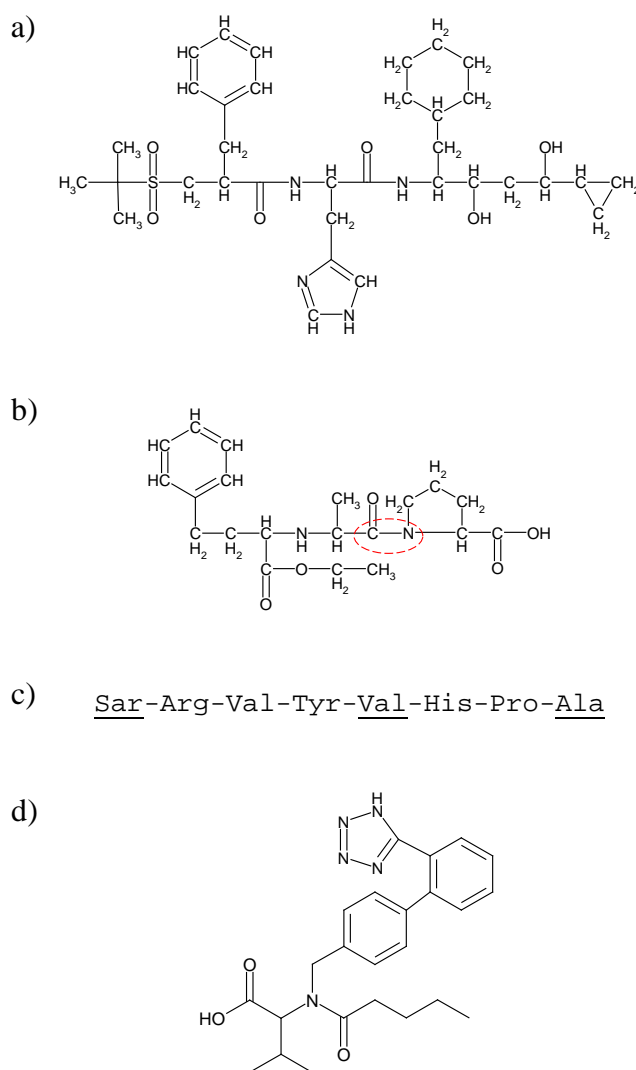


Figure 1.6. Drugs that act on the renin-angiotensin system. a: Remikiren, an inhibitor of renin. Can you see the similarities with the physiological substrate? b: Enalapril, an inhibitor of angiotensin converting enzyme. Enalapril has a higher bioavailability than remikiren does, which is probably related to its smaller size and lower number of peptide bonds. c: Sequence of the synthetic peptide angiotensin antagonist saralasin. Sar = sarcosine (N-methylglycine). Amino acid residues not occurring in angiotensin are underlined. d: Valsartan, an angiotensin receptor antagonist. Note the low degree of similarity with the physiological agonist.

1.6d) is orally applicable, but has very limited similarity to the physiological agonist.

Enalapril and valsartan represent the two practically most important functional groups of drugs, respectively – enzyme inhibitors, and hormone or neurotransmitter receptor blockers. Another important group of drugs that act on hormone and neurotransmitter receptors are ‘mimetic’ or agonistic drugs. However, there is no clinically useful example in the renin-angiotensin pathway; we will see examples later.

1.4. Synthetic drugs may exceed the corresponding physiological agonists in selectivity

Angiotensin is an example of a peptide hormone. Peptide hormones and neurotransmitters are very numerous, and new ones are constantly being discovered, as are new locations and receptors for known ones. While several drugs exist that act on peptide receptors (most notably, opioids), drug development generally lags behind the physiological characterization. The situation is quite different with another group of hormones / transmitters, which are smaller molecules, most of them related to amino acids. With many of these, the availability of drugs has enabled the characterization of different classes of receptors and their physiological roles. The classical example is the distinction of α - and β -adrenergic receptors (which we will consider in more detail later on in this course). While both epinephrine and norepinephrine act on either receptor (though with somewhat different potency), the distinction became very clear with the synthetic analog isoproterenol, which acts very strongly on β -receptors but is virtually inactive on α -receptors (Figure 1.7).

Agonists and antagonists that are more selective than the physiological mediators are both theoretically interesting and of great practical importance. As a clinically significant example of a selective receptor antagonist, we may consider the H_2 histamine receptor in the stomach, which is involved in the secretion of hydrochloric acid (Figure 1.8a). The mediator itself – histamine – was used as starting point in the search for analogs that would bind to the receptor but not activate it. The first derivative that displayed strongly reduced stimulatory activity (while still binding to the receptor, of course) was N-guanylhistamine (Figure 1.8b). Further structural modification yielded cimetidine, which was the first clinically useful H_2 receptor blocker. It represented a major improvement in ulcer therapy at the time and is still in use today, although more modern drugs have largely taken its place.

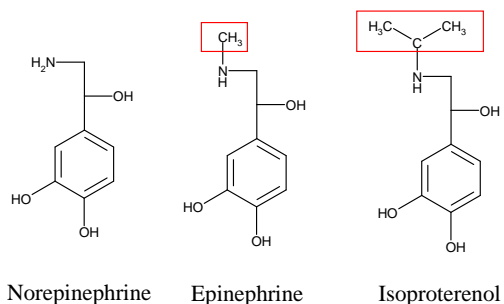


Figure 1.7. Structures of the natural adrenergic agonists, norepinephrine and epinephrine, and the synthetic β -selective agonist isoproterenol.

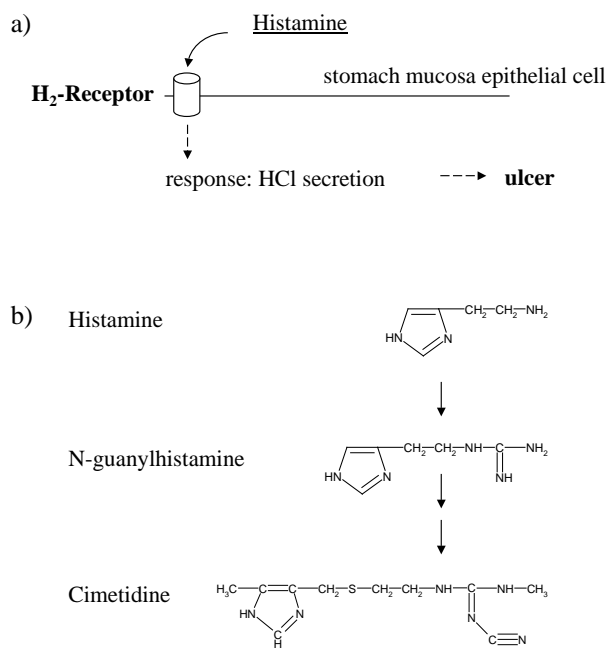


Figure 1.8. Histamine H_2 receptors and receptor antagonists. a: Function of histamine in the secretion of hydrochloric acid from the stomach epithelium. Hypersecretion promotes formation of ulcers. b: Development of H_2 -receptor antagonists by variation of the agonist's structure. Cimetidine was the first clinically useful antagonist.

While H_2 -selective blockers retain some structural resemblance to the original mediator (histamine), the same cannot be said of the likewise clinically useful H_1 blockers, which were developed for the treatment of allergic diseases such as hay fever (Figure 1.9).

Indeed, the H_1 blockers do seem to be plagued by significant 'cross-talk' to receptors other than histamine receptors. This is not uncommon – many agents, particularly those that readily penetrate into the central nervous system, have incompletely defined receptor specificities, although they are usually given a label suggesting otherwise. They are

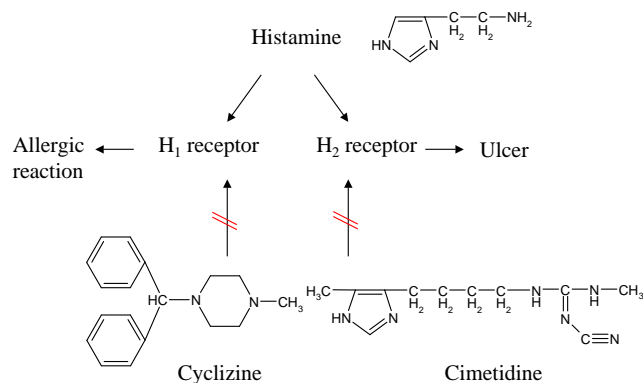


Figure 1.9. Comparison of H_1 and H_2 receptor antagonists. Cyclizine shows very little structural resemblance of the agonist histamine.

frequently used regardless on an empirical basis, often for fairly diverse indications¹.

1.5. Metabolism of physiological mediators and of drugs

So far, we have encountered two reasons for designing drug molecules that are structurally different from physiological mediators:

1. Turning an agonist into an inhibitor, and
2. Increasing receptor selectivity.

Both these reasons relate directly to the interaction of the drug molecule with its target. A third rationale for varying the structure of the drug molecule is that most physiological mediators are rapidly turned over in the organism, which is usually undesirable with drugs. E.g., angiotensin lives only for a few minutes (as does saralasin); the same applies to epinephrine and norepinephrine². With these, one important pathway of inactivation consists in methylation (Figure 1.10).

The drug phenylephrine (Figure 1.10, right) lacks the crucial hydroxyl group that normally initiates inactivation of epinephrine and therefore persists for hours rather than minutes in the organism, making it more practically useful in pharmacotherapy ('take this twice daily with the meal'). Its lower intrinsic affinity to the receptor (about 100fold lower than that of adrenaline) can be offset by increasing the absolute amount applied.

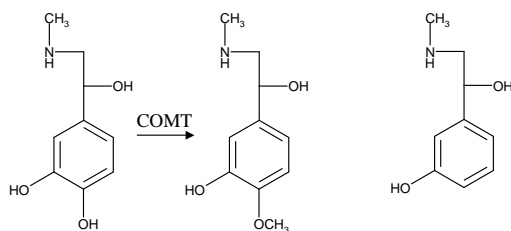


Figure 1.10. Inactivation of epinephrine by catechol-O-methyltransferase. The synthetic adrenergic agonist phenylephrine escapes inactivation because its phenyl ring lacks the 4-hydroxyl group.

¹E.g., H₁-blockers are prescribed to treat insomnia - but I found them not very reliable in this indication. Probably, you have to be driving your car for this to work.

²Notable exceptions are the steroid hormones, which are rather stable; some of these can therefore be directly used for therapy, e.g. hydrocortisone.

In practical pharmacotherapy, a drug's metabolism and elimination are of equal importance as its specific mechanism of action. There are several reasons for this:

1. Drugs may be extensively metabolized in the liver. Since all orally applied drugs are passed through the liver before reaching the systemic circulation, this can lead to impractically low effective levels at the relevant target site. Example: Remikiren (above).
2. Sometimes, the metabolic products are more active than the parent drug, or they may have poisonous effects that were not observed with the parent compound itself³.
3. Diseases – or concomitant use of other drugs – may significantly change the rate of metabolism and thereby change the bioavailability of the drug, leading to loss of desired effects or unacceptably severe side effects.

In the foregoing, we have seen several examples of one frequently used approach to drug development: The structure of a physiological mediator is used as a starting point; a large number of variants are synthesized, and from the pool of variants those with the desired agonistic or antagonistic properties are 'screened' using appropriate *in vitro* assays and animal experiments. This approach does not always work. Below are some examples of other successful approaches to drug development. You will note that some of these are not completely general either.

1.6. Strategies of drug development

Drug development strategies may be classified as follows:

1. Rational design
2. Brute force
3. Traditional medicine / natural products
4. Mere chance.

Note that these distinctions are not really sharp in practice. E.g., the development of H₂-receptor blockers described above would be a mixture of strategies 1 and 2. In reality, one will always try to rationally make use of as much information as possible and then play some kind of lottery to do the rest.

An example of the rational approach to drug design is provided by the development of HIV (human immune deficiency virus) protease inhibitors. HIV protease cleaves viral polyproteins – the initial products of translation – into the individual protein components and thus is essential for

³E.g., prontosil (Figure 1.12) is entirely inactive on bacterial cultures. Only after its reductive cleavage in human metabolism the active metabolite sulfanilamide is released, and antibacterial activity becomes manifest.

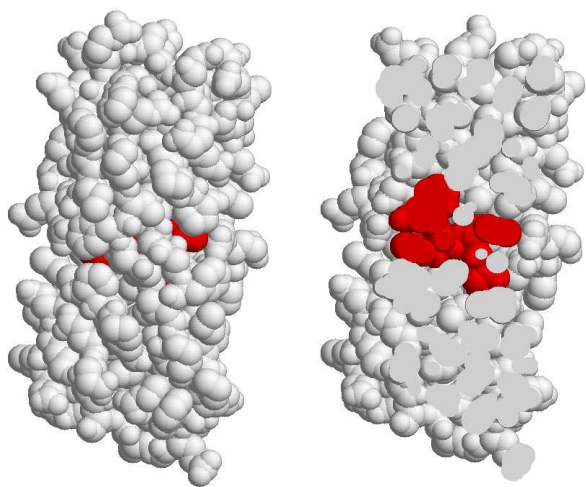


Figure 1.11. Structure of HIV protease, with the inhibitor saquinavir (red) bound in its active site. The sliced view (right) shows the close fit of inhibitor and active site.

the maturation of virus particles. The crystal structure of HIV protease was used to design synthetic molecules that would snugly fit into the active site. Figure 1.11 shows the inhibitor saquinavir bound to the enzyme. HIV protease inhibitors have become one of the mainstays of HIV therapy; their use in combination with reverse transcriptase inhibitors greatly extends the life expectancy of HIV patients.

The brute-force approach involves the following steps:

1. Systematically test every new (or old) compound for drug activity in all kinds of drug activity assays – no matter which purpose it was designed for
2. If you stumble upon something, figure out how it works

A classic success case of the brute-force approach is the discovery of ‘Prontosil rubrum’, the first sulfonamide type

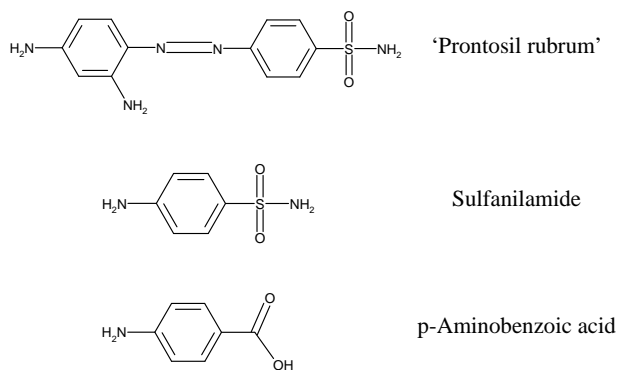


Figure 1.12. Structures of the sulfonamide drug ‘prontosil rubrum’, its antibacterially active metabolite sulfanilamide, and the bacterial metabolite p-Aminobenzoic acid. Sulfanilamide acts as an antimetabolite (i.e., competitive inhibitor) in the synthesis of folic acid, of which aminobenzoic acid is a component.

antibacterial drug (Figure 1.12). ‘Rubrum’ means ‘red’ in Latin – so this is another dye turned drug. The biochemical mechanism was completely unknown by the time, but the drug nevertheless was very active against a considerable range of bacterial species. The discovery of sulfonamides in the 1930s was a major reason for the delay in the development of penicillin, the effect of which was discovered in 1928 but which was not available for clinical use before 1942 (see below).

The brute force approach to drug discovery is still widely used, and one of the reasons why drug design is now largely done by major pharmaceutical companies. In fact, prontosil was discovered at the biggest pharmaceutical company of the era, the German ‘IG Farben’, which was dismantled after the war for its involvement with the production of poisons used in the holocaust.

Traditional medicine is largely based on plants and their various poisons. There is a fair number of drugs originally isolated from plants that are still being used in clinical medicine – even if most of them are now prepared synthetically. This approach may be summarized as follows:

1. Isolate the active components from therapeutically useful and / or toxic plants
2. Elucidate structure, mode of action
3. Find synthetic route, create novel derivatives with improved properties

A classical example is atropine (Figure 1.13). It is isolated from the plant *Atropa belladonna*. ‘Bella donna’ is a common phrase in schmaltzy songs of (true or pretended) Italian origin and means ‘beautiful woman’. In the old days, atropine was used by young women to augment their looks before attending festivities. It widens the pupils of the eyes, and it prevents sweating, therefore leading to accumulation of heat and to red cheeks. At higher dosages, it also caus-

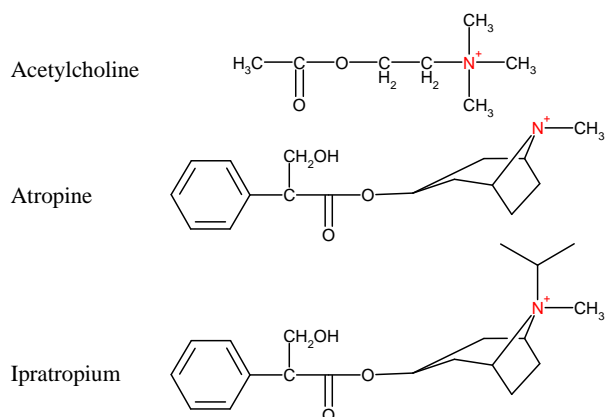


Figure 1.13. Structures of acetylcholine and its competitors atropine and ipratropium. Atropine occurs naturally in *Atropa belladonna*. Ipratropium is a synthetic derivative.

es hallucinations, which may or may not be helpful with falling in love. The hallucinations are, obviously, caused by atropine entering the central nervous system. The central effects are lessened by derivatization of the tertiary amine found in atropine to a quaternary amine, as in ipratropium. Because of its permanent charge, ipratropium does not easily cross the blood brain barrier by 'non-ionic diffusion', and it is therefore often preferred over atropine in clinical medicine.

The final approach to drug development consists in taking advantage of mere chance. The most striking example that comes to mind is the discovery of penicillin. Here is a summary of this 'strategy':

1. Forget to properly cover your petri dish and
2. Have the petri dish contaminated by a mold that kills bacteria (Sir Alexander Fleming, 1929),
3. Wait until somebody else purifies the active ingredient and makes it available for clinical use (Florey and Chain, 1942).

S.A. Waksman took up this paradigm of drug discovery in the 1940's in a more systematic way, starting at stage 2 rather than 1. He succeeded in isolating a large number of antibiotics from a wide variety of soil microorganisms, particularly streptomycetes. The first example was thyrotricin, which is useful for local treatment only. More prominent discoveries of his are streptomycin and chloramphenicol, which can be used systemically and still have their place in therapy today.

(Notes)

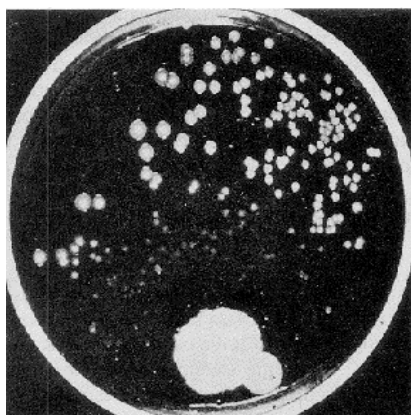


Figure 1.14. The very petri dish that sparked the discovery of penicillin. The white blob at the bottom is a colony of *Penicillium notatum* contaminating a plate streaked with *Staphylococcus aureus* (small, circular colonies). The penicillin diffusing from the fungus radially into the agar has killed off the bacterial colonies in its vicinity.

Chapter 2. Pharmacokinetics

Whatever the actual mechanism of action of a drug may be, we will want to know: Does the drug actually reach its site of action, and for how long does it stay there? This is governed by three factors:

1. Absorption: Uptake of the drug from the compartment of application into the blood
2. Distribution: Transport / equilibration between the blood and the rest of the organism
3. Elimination: Filtration and secretion in the kidneys; chemical modification in the liver

Broadly speaking, absorption and distribution determine whether a drug will be available at its target site at all, while elimination determines for how long the drug effect will last. The issues of drug absorption, distribution and elimination are collectively referred to as 'pharmacokinetics'.

2.1. Drug application and uptake

You are certainly aware that drugs are applied by various routes; the choice depends largely on the pharmacokinetic properties of the drug in question. Table 2.1 lists some characteristics of the major routes.

We will look at the various routes of application in turn. Oral uptake is the most common one, so let's start with this one.

2.1.1. Oral drug application

Inside the digestive tract, drug molecules encounter a quite aggressive chemical milieu. E.g., the acidic pH in the stomach (pH ~2) and the presence of proteases and nucleases in the gut preclude the application of proteins, nucleic acids, and other labile molecules. The gut mucous membrane presents a barrier to uptake; many drugs are not able to efficiently cross it by way of diffusion.

For those drugs that make it from the gut lumen into the blood, the liver presents another formidable barrier. All blood drained from the intestines (as well as the spleen and the pancreas) is first passed through the liver before being released into the general circulation. This is schematically depicted in figure 2.1.

Inside the liver, the blood leaves the terminal branches of the portal vein and the liver artery and is filtered through the liver tissue (Figure 2.3a).

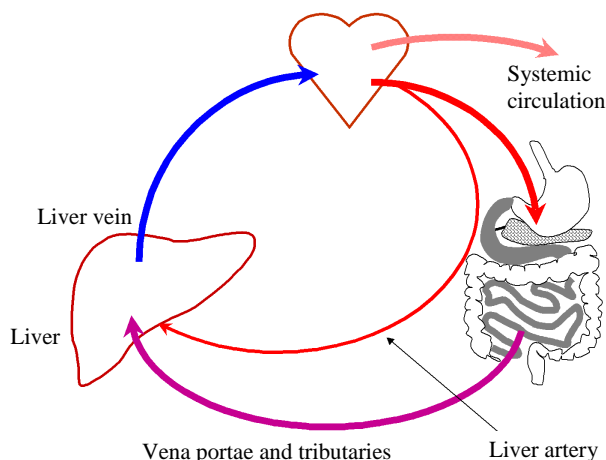


Figure 2.1. Schematic of the portal circulation. Blood drained from all intestinal organs is collected into the portal vein and conducted to the liver. The liver receives an additional supply of oxygen-rich blood via the liver artery.

The liver tissue has a characteristic honey-comb structure (Figure 2.3b). The individual hexagons of the honeycomb are referred to as lobuli. The portal vein and liver artery branches spread along the boundaries of the lobuli. The blood that leaves them is filtered through the tissue towards the center of the lobulus, where it reaches the central vein. The central veins then siphon the blood toward the systemic circulation.

A notable feature of the liver tissue is its lack of real blood vessel walls along the way from the portal vein branches to the central veins. Therefore, the blood gets into intimate contact with the liver epithelial cells, which therefore can very efficiently extract from the blood any compound they see fit (Figure 2.3c).

The liver is a metabolically very versatile organ and is capable of chemically modifying a great many substrates – including drugs – in a variety of ways and with great efficiency. In fact, many drugs cannot be orally applied at all because even during the initial passage the liver extracts them quantitatively from the portal venous blood. This phenomenon is called the 'first pass effect'. An example of a drug that undergoes a substantial first-pass effect is propranolol (Figure 2.2).

Propranolol, which blocks β -adrenergic receptors, is commonly used in patients with cardiovascular disease. Shown below are two metabolites. The left one (4-hydroxypropranolol) is still active but not quantitatively very important. The right one (naphthyloxymethyl lactate) is entirely inac-

| Route | Advantages | Disadvantages |
|-----------------------|--|--|
| Oral | Convenience – route of choice if possible | Multiple barriers and obstacles to efficient uptake into systemic circulation 1. Aggressive milieu in stomach and gut lumen 2. Liver barrier |
| Intravenous | Efficient – quantitative delivery of drug to circulation | Involved – needs skilled labor, risk of infection, enhanced risk of drug allergy |
| Pulmonic (inhalation) | Fast, quantitative uptake | Limited to gaseous agents (oxygen / narcotics) |
| Topical | High drug concentration can be achieved, toxic side effects can be minimized | Limited to accessible sites (skin, mucous membranes) |

Table 2.1. Drug application routes. Note that inhalation of gases is very different from inhalation of aerosols. Gases will, like oxygen, be systemically distributed, whereas the droplets of aerosols will be deposited on the mucous membranes of the bronchi. Accordingly, aerosols are mostly used for topical therapy of asthma.

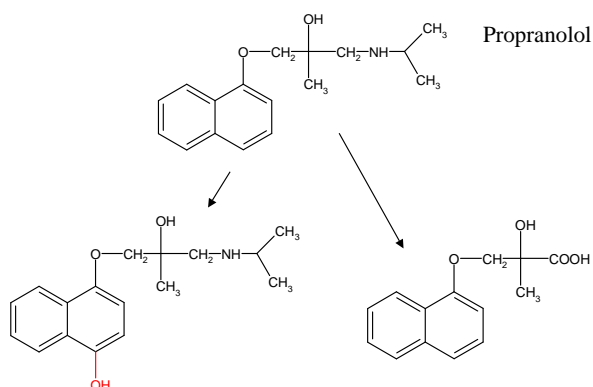


Figure 2.2. Propranolol and two of its major metabolites. The hydroxylated derivative still has β -antagonistic activity. The other compound is inactive.

tive. Only about 30% of the propranolol ingested actually shows up in the systemic circulation – the rest is either not absorbed or metabolized in the liver during the first passage. The extent of this first pass effect shows considerable inter-individual variation – which means that the required dosage may vary considerably and has to be empirically determined with each patient. The fraction that reaches the systemic circulation (~30% in our example) is designated as the ‘bioavailability’ of the drug.

To sum up: Oral application has

- Advantages: Convenience – route of choice if possible
- Disadvantages:
 1. Aggressive chemical milieu in the digestive tract – precludes application of proteins, nucleic acids..
 2. Gut mucous membrane presents a barrier

3. Blood from the intestine is passed through the liver – liver may immediately extract and metabolize the drug (‘first pass effect’)
4. Absorption is slow (not suitable for emergency treatment) and variable

2.1.2. Intravenous drug application

With intravenous application, we have the following advantages:

- ‘Absorption’, even of large molecules, is quantitative and instantaneous. This is essential if drug action is needed immediately.
- Short-lived drugs can be continuously applied by infusion, and the infusion rate can be controlled so as to ‘titrate’ the clinical effect. Examples: Muscle relaxation with succinylcholine during narcosis, control of blood pressure in hypertonic crisis with sodium nitroprusside (both drugs will be discussed later in this class).
- No exposure of drug to harsh conditions – proteins can be applied this way

Disadvantages:

- Involved (needs trained professional for each application – dangerous if not performed properly)
- Adverse reactions to drugs will be more instantaneous and serious, too (example: penicillin allergy)

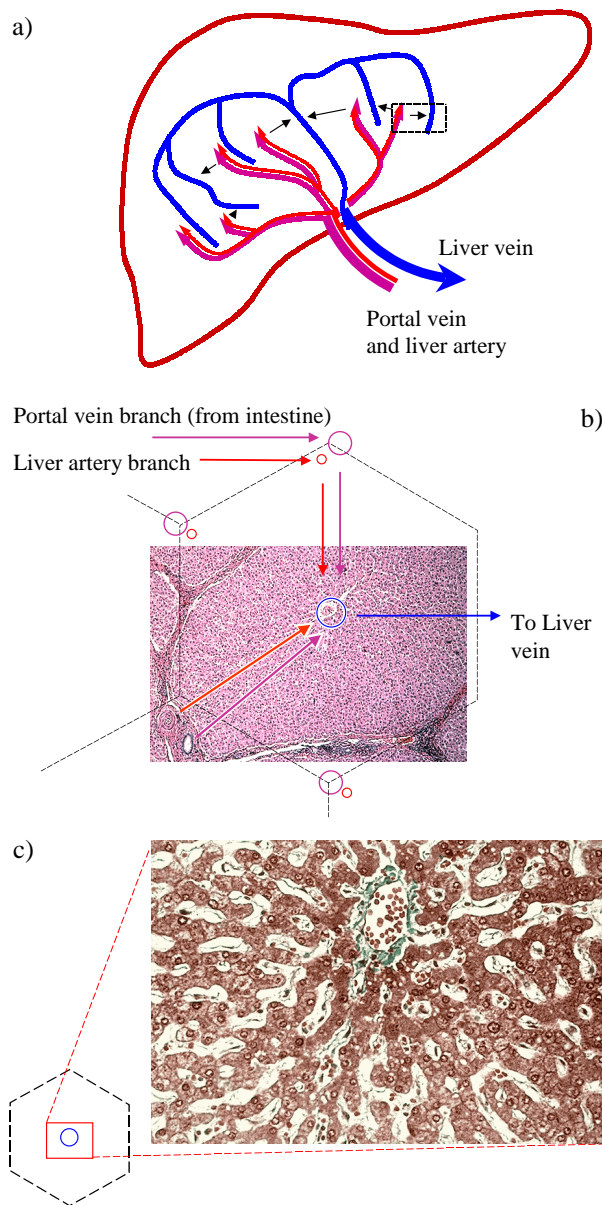


Figure 2.3. Blood circulation and tissue perfusion in the liver. a: Schematic of the blood circulation. Portal vein and liver artery branch out in a parallel fashion. From the terminal branches, the blood enters the tissue and is then collected into the tributaries of the liver vein. b: The liver tissue has a 'honeycomb' structure; each hexagon is a liver lobule. The liver artery and portal vein branches are located at the corners; in the middle of the lobule, we find the 'central vein' which merges with others to form the liver vein. c: Higher power view, showing the sponge-like structure of the liver tissue. The blood gains intimate contact with virtually every liver cell – diffusional barriers are absent, and distances extremely short.

2.1.3. Other routes of drug application

Dermal application has two cases:

- Topical application (treatment of skin disease). No critical issues here; often preferable to systemic therapy

(high local drug concentrations, minimal side effects on the rest of the body).

- Dermal application for systemic use.
 - Uptake typically slow and inefficient (Mother Nature gave us skin as a barrier, not as a conductor). Notable exception: very hydrophobic compounds (organic solvents, nerve gases).
 - Retarded uptake can be utilized for sustaining prolonged, slow delivery (example: Nicotine for weaning smokers)

Mucosal application exploits the fact that, compared to the skin, the barrier is much thinner. Moreover, the veins underlying the mucous membranes in the two favorite places (nose and rectum) are not drained into the liver – i.e., the first pass effect can be circumvented. Examples:

1. Nose: Cocaine, antidiuretic hormone (ADH). ADH is a peptide – so even peptides can make it across the mucosa
2. Rectum: Acetaminophen. Rectal application will increase the bioavailability of this drug as compared to oral uptake, because the first pass effect is absent.¹

Pulmonary application (Figure 2.4) has two modes:

- Gaseous drugs reach the alveoli. This mainly applies to inhalation anesthetics (chloroform, ether, N_2O , and their more modern replacements). Very rapid transition into the bloodstream – very rapid onset of action.
- Non-gaseous drugs can be conveyed by aerosols. The droplets are actually deposited in the bronchi but do not reach the alveoli (topical / mucosal application). Exam-

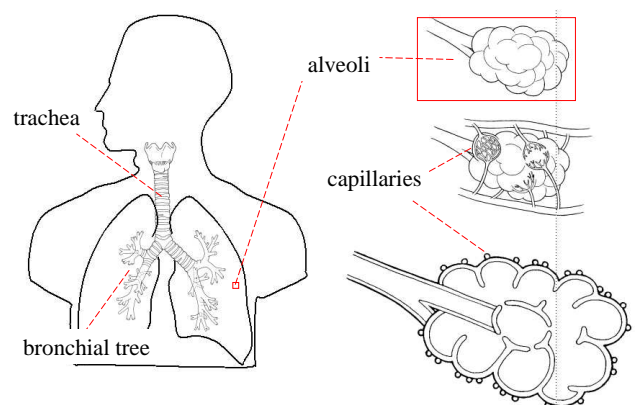


Figure 2.4. Schematic of gas exchange in the human lung. The distance for diffusion is a mere $\sim 20 \mu m$. The total surface area available for exchange is about $80 m^2$. Exchange of oxygen, CO_2 and 'drug' gases such as narcotics is therefore very fast.

¹More precisely, diminished – the rectum is not drained toward the liver at its very end, but a few centimeters above it is.

ple: Steroids for asthma therapy (asthma is an affliction of the bronchi).

Pulmonic absorption is very fast – just like the exchange of oxygen and carbon dioxide. An adult's lung has a full 80 m² of exchange-active area.

2.2. Drug distribution

Once the drug has entered the systemic circulation, it needs to reach its target site. Target sites may be located in various compartments:

1. Within the blood vessels. Example: blood coagulation / clot dissolution. No problems of distribution here, drug molecules of any size and shape can be used (when intravenously applied).
2. In the organ tissue, outside the blood vessels, but extracellular or superficially exposed on the cell surface. Example: Most receptors for hormones and transmitters.
3. In the organ tissue, intracellularly located. Example: Many enzyme inhibitors.

2.2.1. Vascular permeability; the blood brain barrier

An important factor in the distribution of drugs is the permeability of the capillaries. Capillaries are the microscopically small blood vessels across the very thin walls of which metabolites and gases are exchanged between blood and tissues. Capillaries have a cellular layer – the endothelium, supported by a basal membrane consisting of proteins and proteoglycans (Figure microcirculation).

In the general circulation, the endothelial cells have gaps between them (and sometimes fenestrations across individual cells, to the same effect). The permeability then is determined by the sieving properties of the basal membrane, which permits diffusion of salts, small molecules, and even some proteins, although most plasma proteins are retained. This type of capillary does not present a barrier to the distribution of most drugs. However, in the brain and spinal chord (the central nervous system, CNS), the endothelial cells are tightly connected by structures called 'tight junctions' and do not have fenestrations. In addition, a second contiguous cellular layer is formed around the capillaries by the glia cells. This adds up to four cell membranes layered in series – a structure that is referred to as the *blood brain barrier*. Therefore, even small molecules cannot freely migrate into the brain tissue – or only so, if they are extraordinarily membrane-permeant.

Additional cell membrane barriers (plasma membrane, and possibly organelle membranes) will have to be overcome if the drug target is located intracellularly. It thus turns out

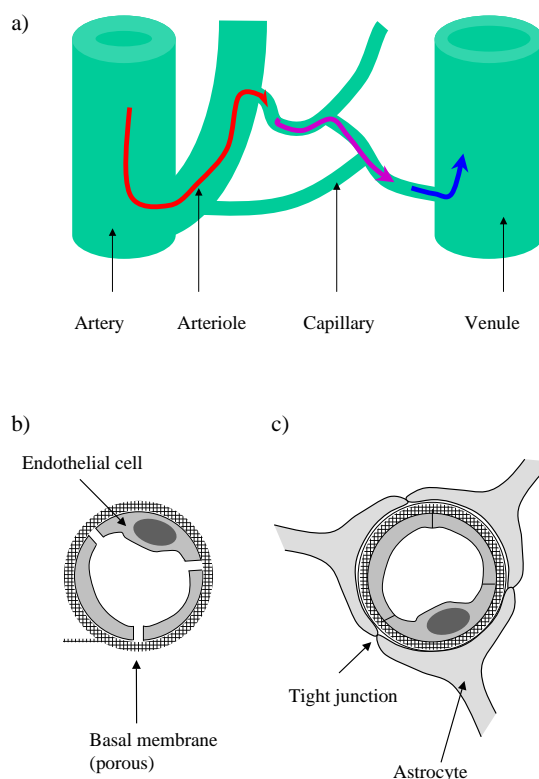


Figure 2.5. Anatomic features of the microcirculation. a: Overview. Arteries branch into arterioles, which are important in the regulation of blood pressure (see later). From the arterioles, capillaries branch off. Here, gas and metabolite exchange takes place; accordingly, capillaries have very thin vessel walls. They empty into venules, which merge into larger veins. b, c: Capillary wall structure. In the general circulation (b), the endothelial cells have gaps between them. The only barrier is the basal membrane, which is readily permeable to small molecules. In contrast, in the central nervous system (c) the endothelium is tightly sealed, and the astrocytes form another tight seal around the exterior circumference.

that cell membranes are of major importance as barriers toward drug distribution.

2.2.2. Drug hydrophobicity and permeation across membranes

Figure 2.6a shows the general structure of a lipid bilayer (yawn). The obstacle to drug diffusion is the hydrophobic core of the membrane. Substances that are lipophilic will traverse the membrane more easily, because they readily partition into this hydrophobic compartment (Figure 2.6b).

The lipid solubility of an organic molecule is influenced in predictable ways by the functional groups it contains. Charged and polar moieties will reduce lipid solubility, and therefore render the drug molecules less membrane-permeant. Fig. 2.7a shows some examples.

To increase lipid solubility, a drug may be applied as a 'pro-drug' that has some hydrophilic groups masked by more

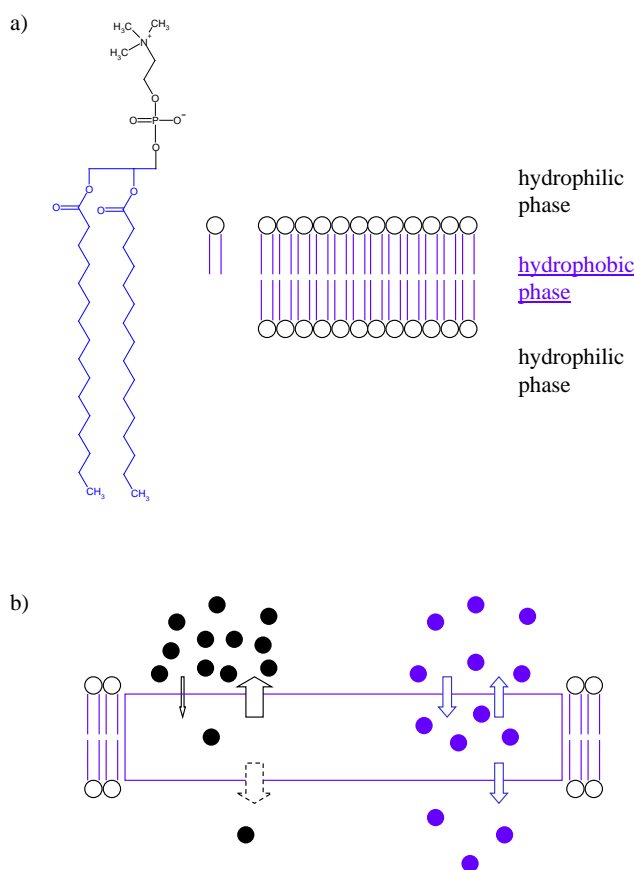


Figure 2.6. The role of lipid membranes in drug distribution. a: Structure of phosphatidylcholine (left), and schematic of a lipid bilayer (right). The hydrophobic interior phase represents the kinetic barrier to drug absorption and distribution. b: Drug diffusion across lipid bilayers. Partition into the bilayer is the rate-limiting step. Hydrophilic drug molecules (left) will not efficiently partition into the hydrophobic phase and therefore can't get across the membrane easily. In contrast, hydrophobic molecules (right) will enter the membrane readily and therefore will cross the membrane more efficiently.

hydrophobic ones. An example is bacampicillin, which is a derivative of the antibiotic ampicillin (Figure 2.7b). Esterification of the carboxylic acid in ampicillin facilitates uptake from the gut lumen. Esterases present in the intestinal mucosal cells will cleave the ester and release ampicillin, which is then passed on into the circulation.

Masking hydrophilic groups also enhances the uptake of drugs into the brain. A classical example is heroin, which is the diacetylated derivative of morphine (Figure 2.7c). Ironically, heroin was invented in an attempt to overcome the addictive effects of morphine. Methadone was later invented to avoid those of heroin.

Another strategy to improve the membrane permeant properties of a drug is based on the effect of 'non-ionic diffusion'. An example is provided by the two 'ganglion-blocking' agents hexamethonium and mecamylamine, which act as antagonists at certain receptors of the transmitter acetyl-

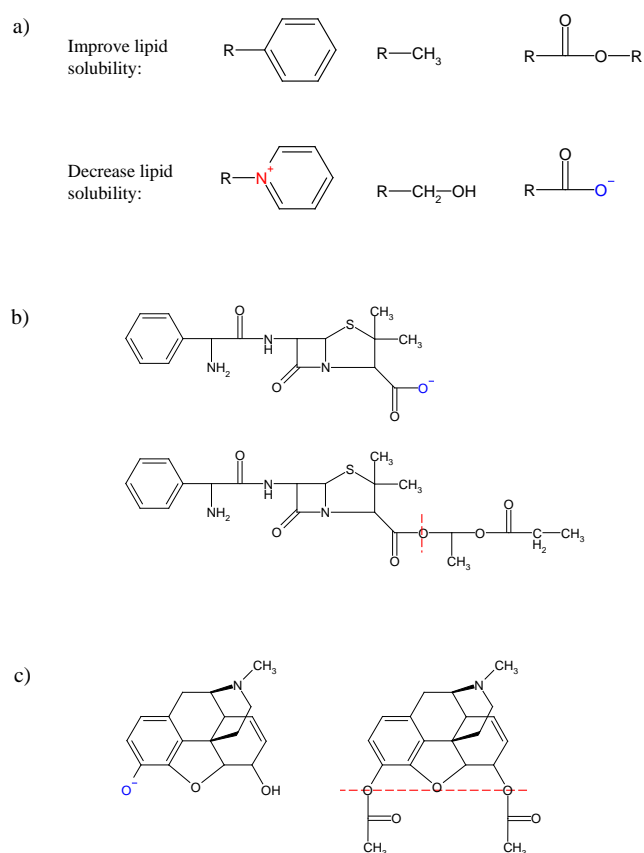


Figure 2.7. The role of functional groups in drug distribution. a: Some functional groups in drug molecules that affect lipid solubility and membrane permeability. b: Ampicillin (top) and its 'resorption ester' bacampicillin (bottom). The pro-drug bacampicillin is cleaved to release ampicillin after intestinal uptake. c: Morphine (left) and heroin (right). The acetyl groups facilitate distribution into the central nervous system, where they will be cleaved off.

choline (Figure 2.8a) and were formerly used as antihypertensive agents. Acetylcholine is a quaternary amine; so is hexamethonium. As a (dual) quaternary amine, hexamethonium is not able to traverse membranes and thus can only be applied intravenously. Mecamylamine, however, is a tertiary amine and can adopt an uncharged (though pharmacologically inactive) form that traverses membranes with ease. Having reached its target site, it can change back into the charged form and exert its effect. It can therefore be orally applied.

Non-ionic diffusion can also produce unwanted effects, as in the case of aspirin (acetylsalicylic acid; figure 2.8b). In the acidic milieu of the stomach, this molecule will be protonated and thus uncharged, which promotes its diffusion into the cells of the stomach mucous membrane. Inside the cell, the pH is very close to neutral, which will lead to deprotonation of aspirin. Diffusion of the deprotonated (charged) form out of the cell will be much slower than entry, so that aspirin will accumulate inside the cells to con-

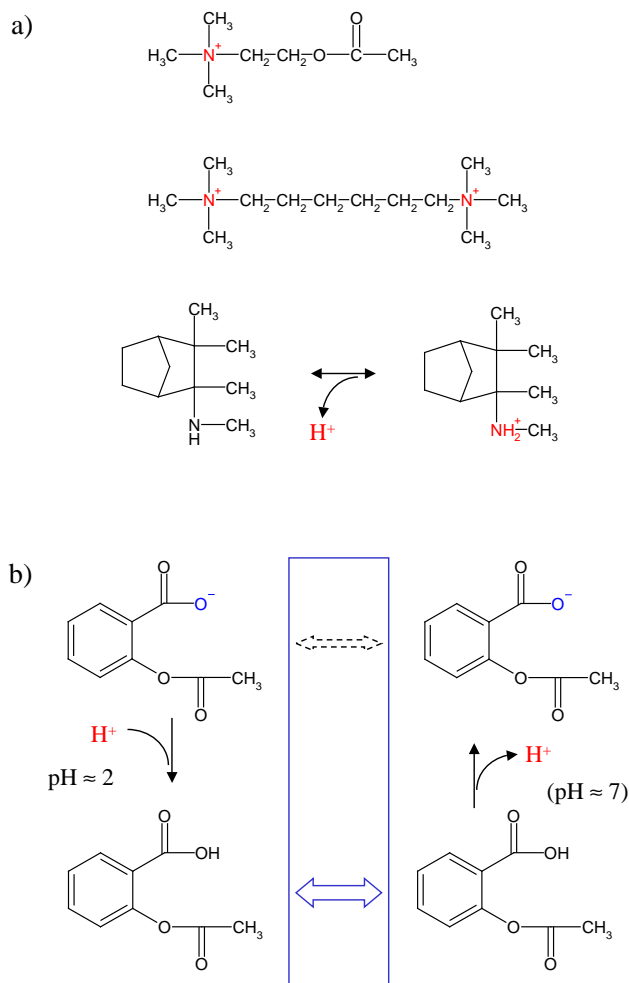


Figure 2.8. Non-ionic diffusion in drug distribution. a: Structures of acetylcholine and of its two antagonists hexamethonium and mecamylamine. Diffusion is facile in the non-ionic form (bottom left), whereas receptor binding requires the positive charge of the protonated state. b: Acetylsalicylic acid is protonated in the acidic milieu of the stomach (left) and then enters the epithelial cells by non-ionic diffusion. Deprotonation at the higher intracellular pH leads to accumulation inside the cells.

centrations considerably higher than in the stomach lumen. Aspirin, compared to other drugs that share its mechanism of action (inhibition of cyclooxygenase; see later), has a stronger tendency to trigger side effects such as gastritis and gastric or duodenal ulcers.

Molecular size is another factor that is relevant to the ease of membrane permeation. This may be illustrated by comparing dimethylether (which crosses membranes readily) to polyethyleneglycol, which may formally be considered a linear polymer of dimethylether (Figure 2.9). PEG is quite efficiently excluded by membranes, particularly in its higher molecular weight varieties. It needs to be pointed out, however, that this example is not entirely valid: PEG is not only larger than dimethylether is but – for some subtle

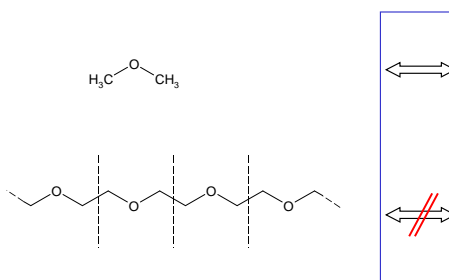


Figure 2.9. Structures of dimethylether and of PEG, which formally (though not in practice) is a polymer of dimethylether. Only the former is membrane-permeant.

reason even our renowned polymer chemist Jean Duhamel was not sure about either – it is also more polar.

2.2.3. L-DOPA as an example of drug distribution facilitated by specific transport

Another strategy to overcome membrane barriers is exemplified by DOPA (dihydroxyphenylalanine), the precursor of dopamine (Figure 2.10). Dopamine is lacking in the brain in Parkinson's disease. If dopamine itself is applied as a drug, it will not be able to cross the blood brain barrier. Although its precursor DOPA is too polar as well to cross the membrane by means of non-specific permeation, it can take advantage of the limited specificity of the aromatic amino acid transporter. This transporter is found in the membranes that make up the blood brain barrier, and its function consists in keeping the brain supplied with phenylalanine, tyrosine, and tryptophan. Evidently, this strategy can be applied only in exceptional cases.

2.2.4. The 'volume of distribution'

After their uptake into the systemic circulation, drugs are distributed between different compartments. These compartments are usually summed up as follows (Figure 2.11a):

The 'interstitial volume' is the extracellular volume outside of the blood vessels. Note that it is three times larger than the intravascular volume! While its ionic composition closely resembles that of blood plasma (with which it is in equilibrium for all small solutes that are not protein-bound), it has a considerably lower protein content.

Body fat is an important reservoir for lipophilic drugs. This volume is more variable than the other ones, so no general volume fraction can be given. However, values in the range of 5-15% are not uncommon.

Few drugs are evenly distributed among these compartments. Factors that will affect the equilibrium distribution include:

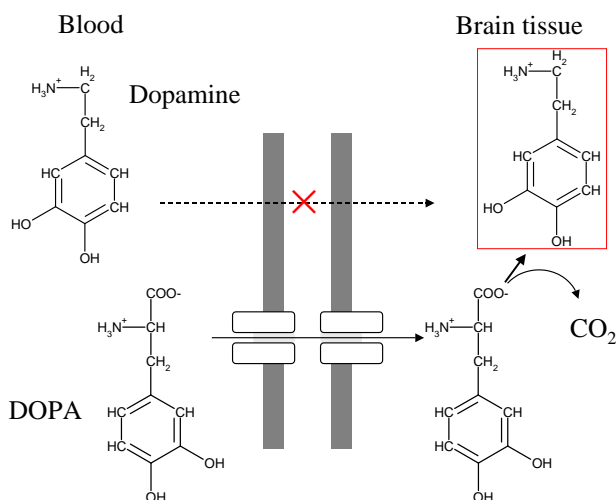


Figure 2.10. Diffusion of DOPA across the blood brain barrier by way of the aromatic amino acid transporter. In the brain, DOPA is decarboxylated to dopamine.

- Membrane-impermeant drugs will be excluded from the intracellular volume (Example: Lithium, which largely resembles sodium in its distribution)
- Lipophilic drugs will be enriched in the fat tissue (example: Thiopental – see later)
- Drugs with a high degree of protein binding will be more enriched in the plasma (i.e., the intravascular volume) than in the interstitial fluid

An uneven distribution between the intravascular and the (combined) extravascular spaces implies that we cannot use the plasma concentration of a drug as an immediate measure of the total amount in the body. To correct for uneven distribution, a coefficient named ‘volume of distribution’ (V_d) has been invented (Figure 2.11a). This is not a real volume but an experimentally determined number (with the dimension of a volume, hence the fancy name).

2.2.5. Protein binding

A factor that favours retention of a drug in the intravascular volume (at least in the short term) is the binding of the drug to proteins (Figure 2.12), particularly albumin. Protein binding is usually more pronounced with hydrophobic drug molecules, which are often bound to > 90% of their total concentration in the blood plasma. Albumin is by far the most abundant single plasma protein. Moreover, each albumin molecule affords multiple drug binding sites; these do not only bind drugs but also fatty acids, which prevents toxic effects of the fatty acids on cell membranes.

Protein binding is usually rapidly reversible, so that the bound fraction is not ‘lost’ – it can yet dissociate and bind to some drug target subsequently. However, one important consequence of plasma protein binding is that it will pre-

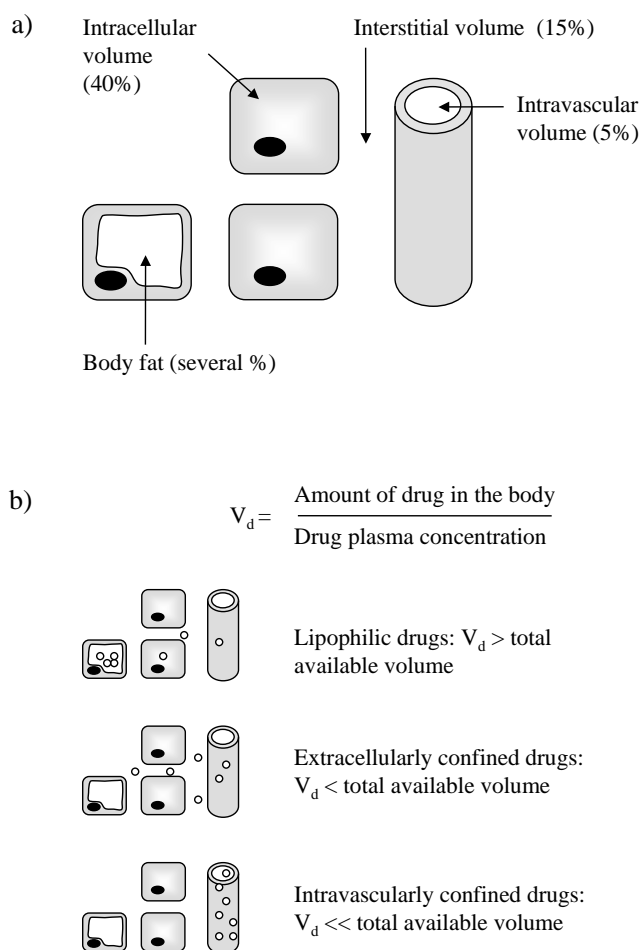


Figure 2.11. a: Compartments of drug distribution. Percentages are relative to total body volume. Note that they don’t add up to 100%, as the volume taken by bone matrix, muscle proteins etc. is not available for solute (drug) distribution. b: The ‘volume of distribution’ (V_d). From its definition, we can see that it will be low for those drugs that are prevented from leaving the blood stream (bottom) or from partitioning into cells (center). It will be very high, often much higher than the real body volume, for lipophilic drugs that accumulate in the fat tissue.

vent glomerular filtration of the drug in the kidneys, which is an important step in drug excretion (see below).

2.2.6. Kinetics of drug distribution

The above considerations on drug partitioning mainly apply to the equilibrium of drug distribution. However, it is important to realize that it may take some time until a drug that is applied rapidly (e.g., by injection or inhalation) actually reaches equilibrium. A practically important example of non-equilibrium distribution is provided by the drug thiopental, which is a barbiturate used for short-duration narcosis (Figure 2.13).

Thiopental is a very lipophilic drug that readily crosses the blood brain barrier. Very shortly after injection, the concentration in the brain peaks, and for a few minutes the

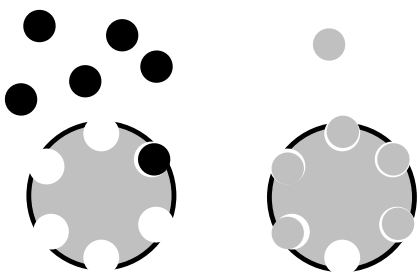


Figure 2.12. Schematic of drugs binding to proteins. Soluble proteins (such as blood plasma proteins) usually have a largely hydrophilic shell with some hydrophobic patches and crevices to which hydrophobic drug molecules will tend to bind. Albumin is the single most important protein contributing to drug binding.

level is high enough to effect narcosis. This is due, among other things, to the fact that the brain receives a very large fraction of the cardiac output (~20%).

However, after a short time, the drug leaves the brain again and accumulates in the lean tissues (such as muscle), from where it finally redistributes to the body fat. This reflects that the fat provides the most favourable (lipophilic) environment; however, since it is only weakly perfused, substance exchange works more slowly than with the other tissues. Note that, in this particular case, drug action is not terminated by elimination of the drug (as is usually the case), but solely by its redistribution from the site of action (the brain) to inert reservoirs (muscle / fat). Ultimate elimination is very slow – it takes days to complete – and involves hepatic metabolism of the drug, followed by urinary excretion.

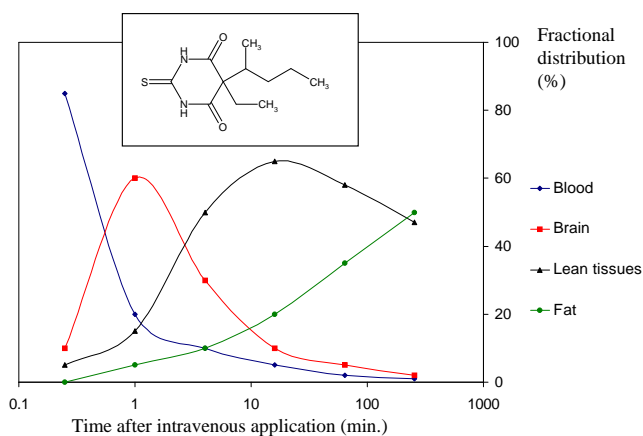


Figure 2.13. Kinetics of thiopental distribution. Thiopental is a very hydrophobic barbiturate that is used for transient narcosis. Duration of the narcosis is limited by redistribution of thiopental from the brain to other body compartments (which is very fast) rather than elimination of the drug (which is very slow).

2.3. Drug elimination: Kidneys

Ultimately, most drugs are eliminated from the body via the kidney. As a rule of thumb, drugs can be directly eliminated there if they are hydrophilic; hydrophobic drug molecules are typically metabolized to more hydrophilic derivatives in the liver before elimination (Figure 2.14).

To understand drug elimination in the kidney, we first have to consider some aspects of its structure and function.

2.3.1. Kidney anatomy and function

The kidneys are located close to the aorta (Figure 2.15a) and, in terms of blood flow / tissue mass, are the most strongly perfused organ. Urine is ‘distilled’ from the blood in several stages:

1. **Filtration:** The kidneys are perfused at a rate of ~1.2 l/min. Approximately 10% of the blood plasma volume is squeezed across a filtering membrane that retains most macromolecules but lets through small molecules.
2. **Re-absorption:** Most small solutes – glucose, salts, and amino acids – are recovered from the filtrate and shuttled back into the blood by specific transporters. Water is recovered by the ensuing osmotic gradient. Some solutes are partially or totally excluded from reuptake.
3. Some substrates are actively secreted from the blood into the nascent urine.

The kidney tissue has a very intriguing structure. It is organized into several thousand structural and functional units. A single unit – a ‘nephron’ (Figure 2.15b) – spans the better part of the entire distance between the organ periphery and the renal pelvis, which simply collects the final urine and feeds it into the ureters..

Urine production starts in the glomerulus (Figure 2.16a, b). Arterial blood is passed along a flexuous stretch of specialized small arteries, the walls of which act as a sieve.

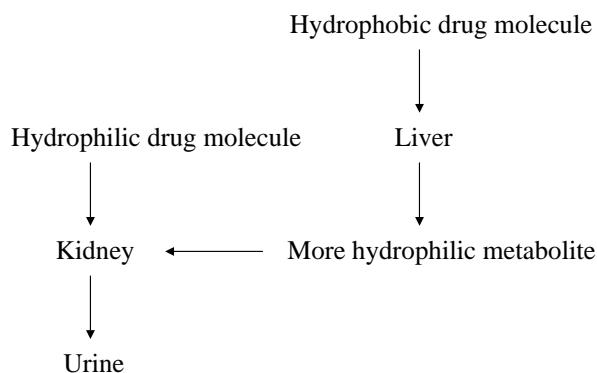


Figure 2.14. Typical pathways of elimination of hydrophilic and hydrophobic drugs

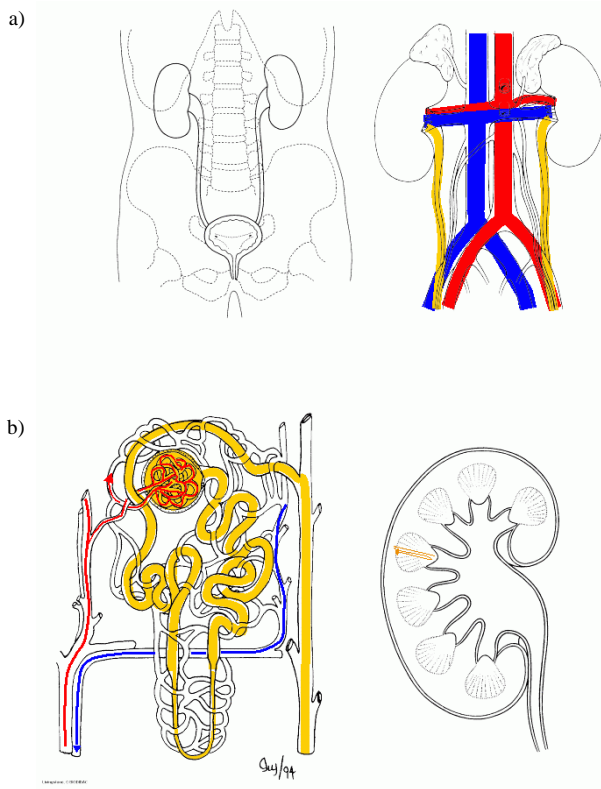


Figure 2.15. Kidney anatomy. a: Overview of kidney and urinary tract. Left: Position of the kidneys, ureters, and urinary bladder within the body. Right: The kidneys are connected to the aorta (red, center, vertical) and the vena cava (blue, center, vertical) by short, wide blood vessels and are strongly perfused. The ureters (yellow) transport the urine to the urinary bladder. b: The nephron. Left: Structural elements of the nephron. The yellow blob with red lines (arterioles) is the glomerulus, which gives rise to a tubule that has convoluted and straight sections and empties into a collecting duct. Center: A single nephron, superimposed on the longitudinal section of a kidney. Right: The true proportions - the nephron has a very elongated shape; the straight section (which is crucial in urine concentration) is very long.

Figure 2.16b shows the structure of the glomerular vessel wall. The interior is covered by endothelial cells with multiple holes ('fenestrations'). The podocytes (= 'foot cells') form a likewise discontinuous outer layer. Between them is an acellular basal membrane, consisting of proteins and proteoglycans, which has the smallest pore diameter of all three and therefore, as in any the capillaries found elsewhere in the body, represents the effective filter layer. The filter has a cut-off size of very few nanometers, so that most protein molecules will be retained. Salt ions and small molecules – if they are not protein-bound – will be filtrated. The amount of filtrate produced is about 150 l per day in a healthy adult; this corresponds to about 1/10 of the blood plasma volume that passes the kidneys.

The filtrate is funnelled into the tubule that leaves the glomerulus and passed down all the tubular elements of the

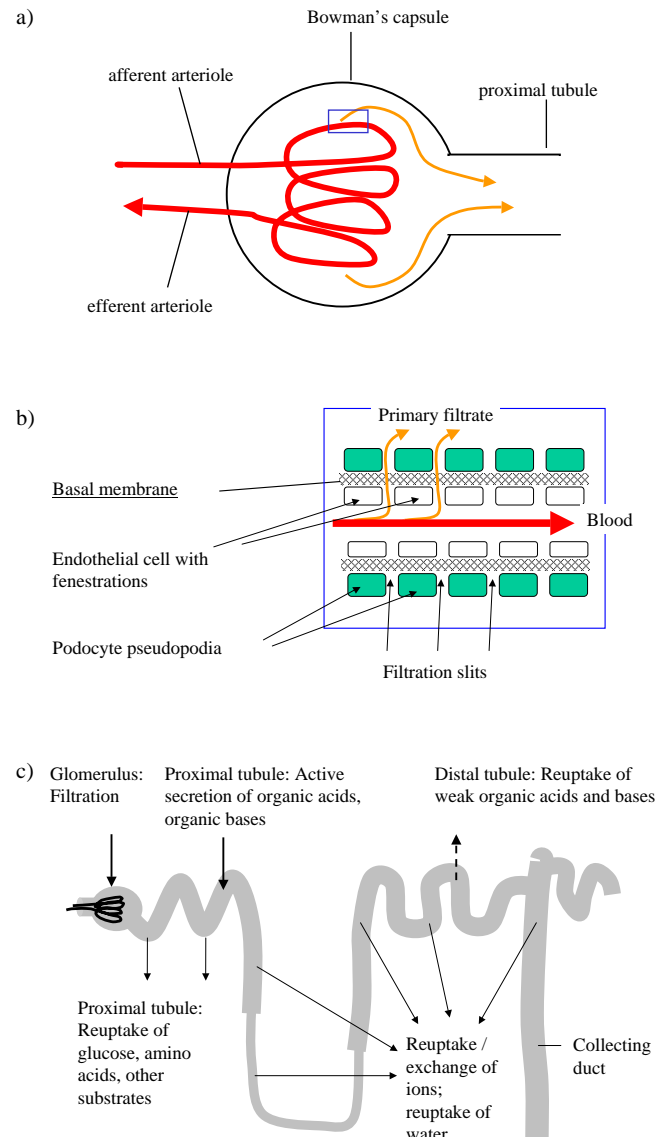


Figure 2.16. Nephron function. a: Filtration occurs in the glomerulus. The filtrate is funneled into the tubule. b: Schematic of the blood vessel wall structure in the glomerulus. Both the endothelium within and the podocytes outside the arterioles have slits and fenestrations that are a few nanometers wide. As in the capillaries elsewhere in the body, the basal membrane functions as the sieve. c: In the tubule and the collecting duct, the filtrate is extensively post-processed; water, substrates and ions are re-absorbed but also actively secreted and exchanged. Tubular processing is under hormonal control.

nephron (see Figure 2.15c). It is during this passage that the volume of the filtrate is trimmed down to the final urine volume, and the urine composition is changed and adjusted in accordance with the prevailing physiological situation. This filtrate post-processing involves both re-absorption and active secretion by the epithelial cells in the tubuli (Figure 2.16c).

These occur at different segments of the nephron:

1. Proximal tubule: Reuptake of glucose, amino acids, bicarbonate; active secretion of uric acid, organic acids, organic bases (including many drugs).
2. Loop of Henle: Reuptake of salt and water.
3. Distal tubule / collecting duct: Reuptake of salt and water; adjustment of pH and ion concentrations to meet physiological needs; passive reuptake of weak acids and bases (including drugs).

Mechanistically, most small solutes – glucose, salts, amino acids – are taken up again by specific active transporters. Active secretion likewise works by way of active transport. Typically, one transporter will pick up the substrate in question from the interstitial space and move it to the cytosol, from where a second transporter located in the apical membrane expels it into the nascent urine (see Figure 2.19). Water is recovered by the ensuing osmotic effect. Some solutes are partially or totally excluded from reuptake. Note that the final urine volume is about 100 times smaller than the primary filtrate. This means that the bulk of the fluid, salt and metabolites are actually reabsorbed. Some drugs are subject to reuptake to a similar extent, too.

2.3.2. Filtration, secretion, reuptake

For a solute (drug) that is quantitatively filtrated in the glomerulus, the extent of excretion is determined by its membrane permeability (Figure 2.17). If the solute is not membrane-permeant, it will get more and more concentrated as the volume of the nascent urine gets reduced along the tubule; however, the absolute amount of the solute retained will not change. A model compound exemplifying this behaviour is inulin, a polysaccharide of about 6000 Da (Figure 2.18). Conversely, a drug that is fairly membrane-permeant (such as ethanol) would just diffuse back into the tissue (and, from there, the circulation). Its concentration in the nascent urine would, at all times, remain in equilibrium with the interstitial fluid (which means, constant); the amount of drug retained in the urine would therefore decrease in proportion to the urine volume. It is for this reason that ethanol is not eliminated efficiently by the kidneys but rather more slowly by the liver. We might pause a moment to lament this, although the high taxes in Canada suggest otherwise.

Membrane-permeant drugs are thus not efficiently eliminated in the urine, even if they do get filtrated in the glomeruli. On the other hand, membrane-impermeant drugs get eliminated in proportion to the extent of glomerular filtration. Glomerular filtration therefore is an important parameter in the elimination of drugs. It may vary considerably between different patients (example: A patient who has donated one kidney. Not the most common case of reduced kidney function but a straightforward one). With

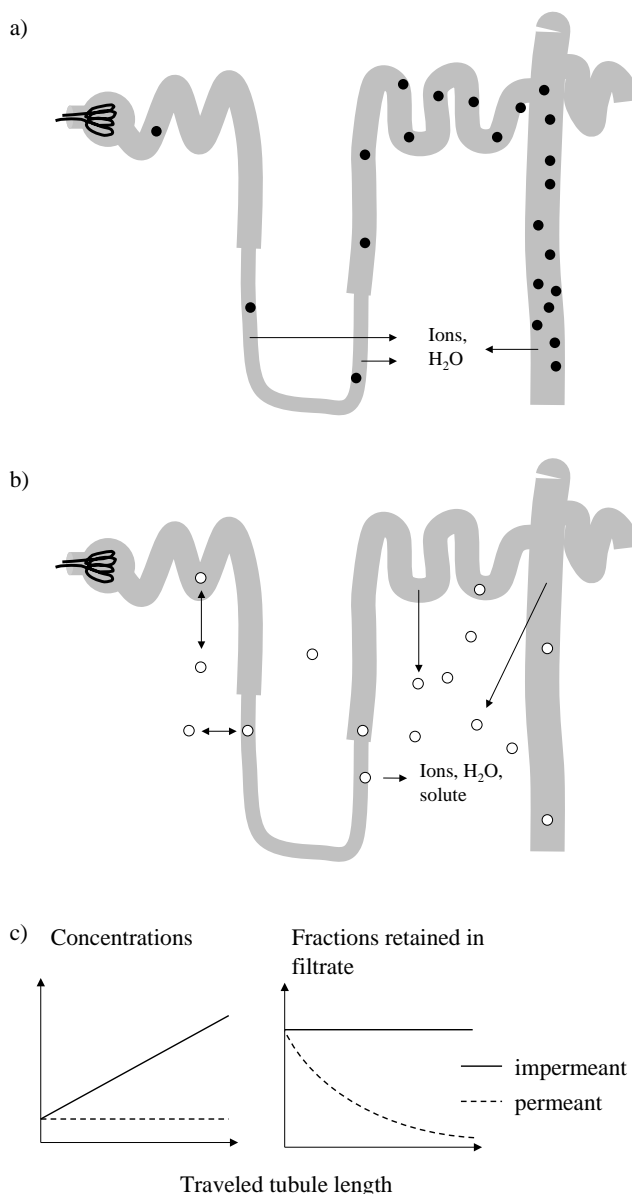


Figure 2.17. The effect of membrane-permeability of drugs on their reuptake from nascent urine. a: A membrane-impermeant solute such as inulin will become more and more concentrated as the urine volume decreases due to water reuptake. Ideally, the fraction retained in the urine will at all times remain at 100% (c, left). b: A membrane-permeant solute such as ethanol will simply equilibrate between tubulus and interstitial fluid; its concentration in the urine will ideally be constant, and the fraction retained will decrease in proportion to the urine volume (c, right). c: Ideal curves for urine concentrations and retention fractions for membrane-permeant and -impermeant drugs.

some drugs, it is important to know the glomerular filtration rate in advance to their clinical application.

An elegant experimental method for its determination uses inulin (Figure 2.18). Here is how this methods works:

Inulin is freely filtrated in the glomeruli, so that the concentration in the filtrate equals that in the plasma:

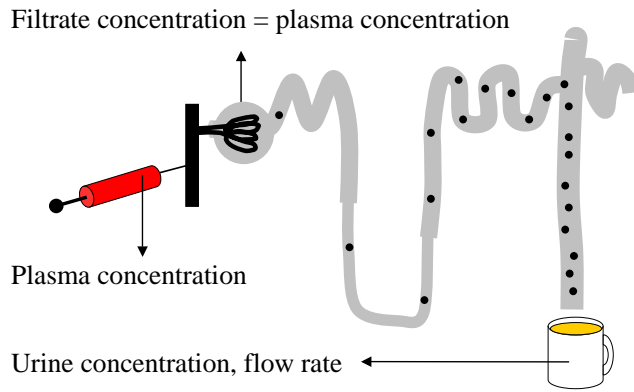


Figure 2.18. Determination of the inulin clearance. Inulin is injected intravenously (ideally by way of continuous infusion), and its concentrations in blood and urine are determined. The ratio of these concentrations will be inversely proportional to the urine volume reduction after glomerular filtration; multiplied by the urine flow, it thus provides an estimate of the glomerular filtration rate.

$$(1) c_{\text{filtrate}} = c_{\text{plasma}}$$

Inulin is quantitatively retained in the urine, so that the number of molecules is the same in the filtrate and the final urine:

$$(2) n_{\text{filtrate}} = n_{\text{urine}}$$

The number of molecules is the product of concentration and volume:

$$(3) c = n/V \Leftrightarrow n = c \times V$$

therefore, with equation 2:

$$(4) c_{\text{urine}} \times V_{\text{urine}} = c_{\text{filtrate}} \times V_{\text{filtrate}}$$

From equations 1, 3 and 4, we see:

$$(5) V_{\text{filtrate}} = V_{\text{urine}} \times c_{\text{urine}} / c_{\text{filtrate}}$$

Therefore, all we need is to apply inulin to a patient by intravenous infusion, collect the urine for a certain amount of time (typically 24 h), determine the urine and plasma concentrations, and apply equation 4 to calculate the volume that has been filtrated during these 24 hours.

The parameter determined in this experiment:

$$V_{\text{urine}} \times c_{\text{urine}} / c_{\text{plasma}}$$

is called the renal 'clearance' of inulin. It can of course also be determined for other solutes. In clinical practice, the endogenous marker creatinine (a metabolite of creatine, from muscle tissue) is commonly used instead of inulin. Its characteristics with respect to secretion and retention are less clear-cut than those of inulin; its clearance therefore is a less accurate measure of the glomerular filtration rate. As the basis of an even less accurate estimate, the plasma concentration of creatinine alone is frequently used, without any actual measurements of urine volume and concentration; the reasoning behind this is that the amount of creatinine produced does usually not vary all that much. This estimate is then used for determining initial drug dosages, which may be adjusted according to assays of the plasma concentrations of the drug itself later on.

Another model substance that is used experimentally for the assessment of kidney function is para-aminohippuric acid (p-AH). p-AH appears in the urine not just by filtration but mainly by active secretion in the proximal tubule. This active transport process occurs in two steps (Figure 2.19a): In the first step, p-AH is exchanged at the basolateral membrane of the proximal tubule cell against α -ketoglutarate or other divalent anions. This exchange is driven by the membrane potential (the interior of the tubule cell is electrically negative relative to the outside, as is the case with essentially all cells).

In the second step, p-AH is secreted from the tubule cell into the tubule lumen. This involves exchange with monovalent anions from the filtrate, driven not by charge but by concentration gradients.

Since p-AH is nearly quantitatively extracted from all blood plasma that reaches the kidney (the commonly reported fraction is 92%), its clearance can actually be used to determine the renal flow of blood plasma, without any serious invasive action. Here is the rationale:

If a certain volume of blood passes through the kidneys, p-AH is quantitatively transferred from the blood plasma to the (nascent) urine:

$$(6) n_{\text{plasma (before passage)}} = n_{\text{urine (after passage)}}$$

With equation 3, we get

$$(7) c_{\text{urine}} \times V_{\text{urine}} = c_{\text{plasma}} \times V_{\text{plasma(kidney)}}$$

with From equations 6 and 7, we see:

$$(8) V_{\text{plasma(kidney)}} = V_{\text{urine}} \times c_{\text{urine}} / c_{\text{plasma}}$$

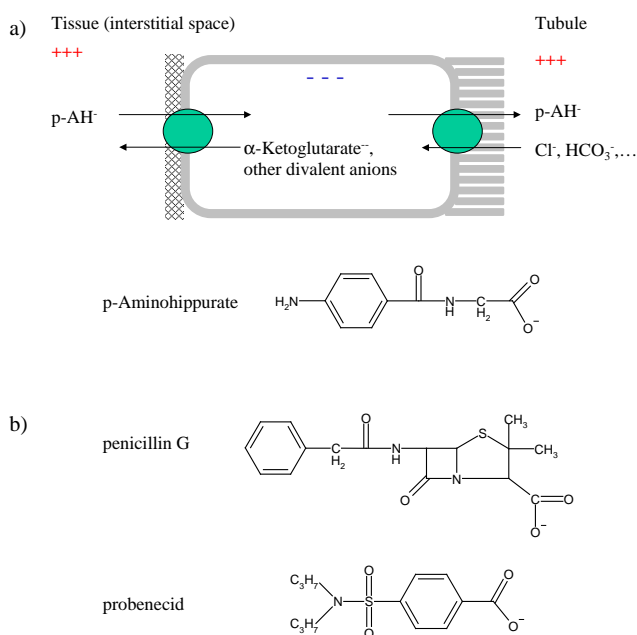


Figure 2.19. Active secretion of organic acids in the proximal tubule. a: Secretion of p-Aminohippurate. Two exchange transporters are involved. The first one is located in the basolateral membrane; its operation is electrically driven. Transport by the second one, which is located in the apical (luminal) membrane, is driven by concentration gradients. This transporter is inhibited by probenecid. b: Penicillin G, like p-aminohippurate, is a substrate for both transporters. Probenecid inhibits the apical transporter and therefore prevents the rapid elimination of penicillin.

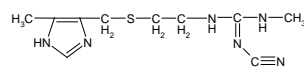
Another implication of the quantitative extraction of p-AH is that this secretion mechanism is very powerful indeed. It is also of low specificity and operates likewise on many drugs that are organic acids, including penicillin.

2.3.3. Examples

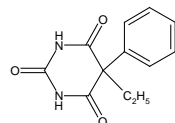
Since penicillin is a substrate for the p-aminohippurate transporters, it is very rapidly cleared from the circulation. In the early days, when penicillin was very expensive, this rapid clearance was a major problem. The urine of patients receiving penicillin therapy was actually collected, and the secreted penicillin recovered. This problem was overcome by the development of probenecid (Figure 2.19b), which inhibits the second step in the above transport process. This results in a very pronounced prolongation of the retention of penicillin in the body. While no longer used routinely, probenecid is still used occasionally if high, stable plasma levels of penicillin are important in the treatment of life-threatening infections, such as brain abscesses.

As pointed out above, the extent of a drug's reuptake in the distal tubule depends on its membrane permeability. Here are two real world examples (Figure 2.20):

| | Protein-bound (%) | Clearance (L/h) | Half-life (h) |
|---------------|-------------------|-----------------|---------------|
| Cimetidine | 19 | 32 | 2 |
| Phenobarbital | 51 | 0.25 | 98 |



Cimetidine



Phenobarbital

Figure 2.20. Pharmacokinetic parameters and structures of cimetidine and phenobarbital

Cimetidine has several ionizable groups and therefore is quite polar. Accordingly, it is only weakly protein-bound, effectively filtrated and retained and achieves a high clearance. Its clearance is actually higher than that of inulin – indicating that it must be actively secreted as well, and thus that active secretion is not confined to acids. Phenobarbital is quite apolar (although it is a weak acid – where is the dissociable proton?). It is only moderately protein-bound; hence, it should get filtrated to about 50%. Yet, its clearance is very low – a clear indication that it gets reabsorbed along the way down the tubule.

An important consideration in this context is that the extent of retention may vary with the urine pH, if the drug molecule is a weak acid or base. An example of applied pharmacokinetics from the underground is LSD (lysergic acid diethylamide; Figure 2.21). While a powerful hallucinogen, it is allegedly quite unpredictable whether the hallucinations will actually turn out pleasant or more along the lines of Count Dracula. In the latter case, it has been recommended to follow up the LSD with lots of vitamin C (ascorbic acid).

The kidneys will excrete excess acid equivalents in the urine. At acidic pH, LSD will become protonated and therefore no longer slip back across the tubule epithelium into the circulation; this will lead to accelerated elimination of LSD. We here have another example of the principle of 'non-ionic diffusion', which we have previously discussed in the context of drug absorption.

The same strategy – artificial alkalization or acidification of the urine – is quite commonly employed in the clinical treatment of poisonings. However, if the poison (drug) is neither acidic nor basic, the only option is to increase the urine volume. In this case, the amount of the drug (assuming it to be membrane-permeant, as many are) eliminated will simply be proportional to the volume of urine produced. This strategy is called 'forced diuresis'. Another, more effective but also more involved method for the accelerated elimination of hydrophobic drugs such as barbi-

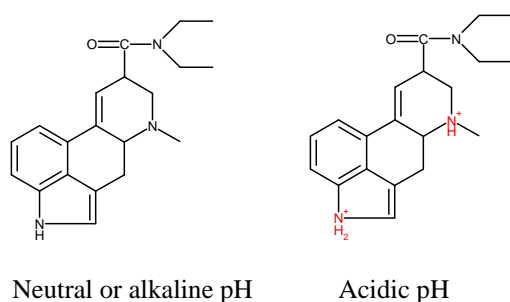


Figure 2.21. Non-ionic diffusion in drug elimination - LSD as an example.

turates is hemoperfusion. Here, blood is diverted from a large artery, typically in the thigh, passed over a hydrophobic solid-phase absorber, and fed back into the corresponding vein.

2.4. Drug elimination: Metabolism

While many drug molecules can be eliminated directly via the kidney, we have seen that others, predominantly hydrophobic ones, do not get efficiently secreted in the urine, be it because of plasma protein binding or because of reuptake in the distal tubule. Even with some of those drugs that are amenable to renal elimination, metabolism may occur and give rise to changes in drug efficacy or to toxic side effects. Drug metabolism happens largely in the liver.

Drug metabolism is commonly – and somewhat arbitrarily – subdivided into phase I and phase II reactions. In Figure 2.22, phase I would correspond to the conversion of a drug molecule to a more hydrophilic metabolite. The latter may then either be directly excreted in the urine, or undergo conjugation with a larger polar moiety before excretion.

2.4.1. Example: Metabolism of phenobarbital and of morphine

We have seen above that phenobarbital is not efficiently eliminated in the urine. It therefore is a good candidate for elimination by hepatic metabolism. The molecule does not have any good functional groups that could serve as points of attachment for glucuronic acid or other polar moieties. Therefore, phenobarbital first has to undergo a hydroxylation reaction before conjugation may occur – an example of a phase I reaction. Conjugation may then occur either with glucuronic acid, or with sulfate (Figure 2.23a). Either modification will inactivate the molecule and render it suitable for renal excretion. The glucuronide may also be excreted in the bile.

However, some drugs may not undergo a phase I reaction at all but undergo conjugation directly. An example is provided by morphine². Morphine has two free hydroxyl groups,

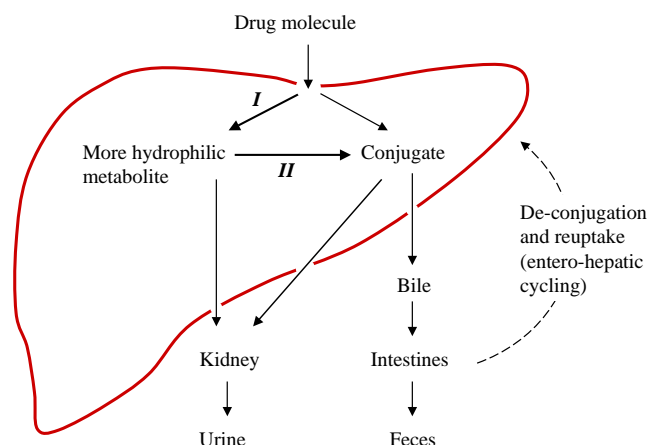


Figure 2.22. Outline of hepatic drug metabolism and its role in drug elimination. I, II: Phase I and phase II reactions. Some drugs skip the phase I reaction and are directly conjugated. Metabolites may be either released into the blood stream and eliminated by the kidneys, or they may be secreted into the bile. In the latter case, deconjugation may occur in the intestine (largely due to bacterial enzymes), and the drugs released may undergo 'entero-hepatic cycling'.

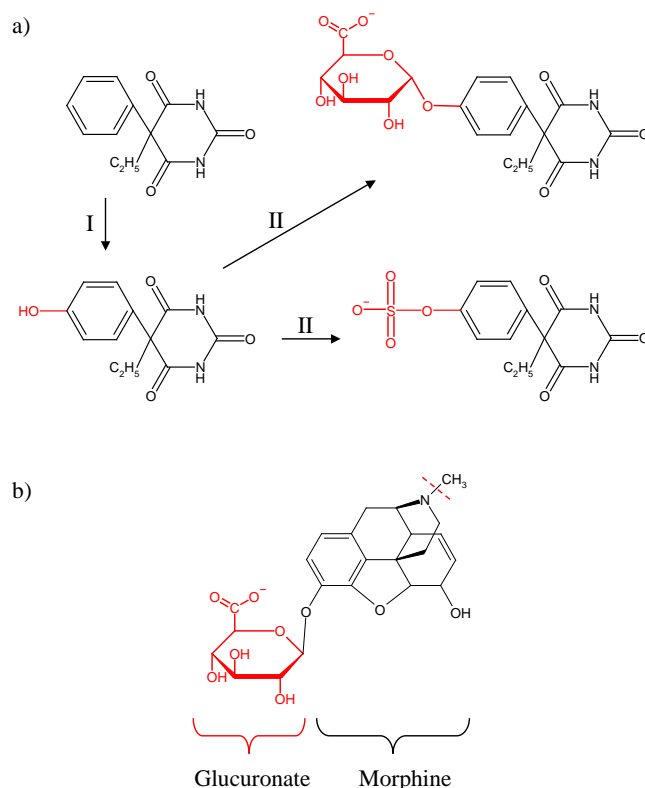


Figure 2.23. Metabolism of phenobarbital and of morphine. a: Two-stage metabolism of phenobarbital. The initial hydroxylation (phase I) creates the anchor for attachment of polar moieties, in this case glucuronic acid (right, top) and sulfate (right, bottom). b: Morphine has free OH groups to start with and therefore does not require a phase I reaction. However, in addition to conjugation, desmethylation may occur.

²You may recall that heroin is a diacetylmorphine. Heroin would have

to either or both of which a UDP-glucuronosyltransferase in the liver ER will attach a glucuronic acid moiety.

The conjugates formed with glucuronic acid are called *glucuronides*, not glucuronates, because the bond created is a glycosidic bond but not an ester bond. The carboxylic acid group remains free and contributes to the overall hydrophilicity (Figure 2.23b).

2.4.2. Cytochrome P450 enzymes

Enzymes of the cytochrome P450 family are responsible for most phase I reactions. Cytochrome P450 enzymes are extremely widespread in nature, and they occur in both prokaryotic and eukaryotic cells. In eukaryotic cells, these enzymes mostly reside in the membrane of the smooth endoplasmic reticulum, but some variants are found in the mitochondria.

A cytochrome P450 enzyme works in conjunction with a reductase, which supplies it with electrons from NADPH (and uses FAD and FMN sequentially in the electron transfer process). The two electrons are delivered to the heme cofactor in the active center of the cytochrome, which in turn transfers them to one of the two oxygen atoms of O_2 to yield water (Figure 2.24). Presumably, the free energy of the oxidation of NADPH is somehow utilized to facilitate the reaction of the other oxygen with the organic substrate. This may result in the formation of a phenolic hydroxyl group, as in the case of phenobarbital. However, the oxygen may react with the substrate in various ways:

- N-dealkylation (Figure 2.25a; also see Fig. 2.23b)
- O-dealkylation (Figure 2.25a)
- N-oxidation and N-hydroxylation (Figure 2.25b)
- Sulfoxide formation (Figure 2.25c)

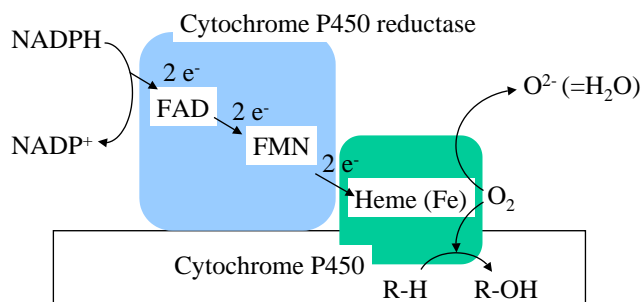


Figure 2.24. Overview of the membrane-associated cytochrome P450 system. Hydrogen is abstracted from NADPH and used to reduce one atom of molecular oxygen to water; the second oxygen atom reacts with the substrate, often forming a hydroxyl group.

to be deacetylated first before glucuronidation; this happens in both the liver and the brain. This deacetylation step could be classified as a phase I reaction.

- Oxidative deamination (Figure 2.25d)
- Formation of epoxides from aromatic precursors. This reaction may actually be quite harmful. Epoxides are highly reactive and can do a lot of damage in the cell (more on this below).

The effects of cytochromes P450 in drug metabolism are thus quite varied, and they involve numerous enzyme species. However, it is noteworthy that one individual enzyme—named CYP3A4—participates in the conversion of up to 60% of all drugs that do get metabolized. While CYP3A4 is always present to some extent, the activity can be substantially increased by a variety of drugs by a process called enzyme induction. Basically, induction of drug-metabolizing enzymes works like the good, old lac operon in *Escherichia coli* (Figure 2.26): The drug enters the cytosol and associates with a protein receptor molecule named pregnane X receptor (PXR) which is homologous to a number of endogenous hormone receptors, many of which bind steroid hormones.

Upon drug binding, this receptor translocates to the nucleus, associates with some more proteins (including hnf4) and binds to specific sites in the DNA to up-regulate several genes, including CYP3A4. Interestingly, it also induces membrane transporters such as P-glycoprotein that are involved in excretion of metabolites from the cell.

PXR has a remarkably broad specificity, including both endogenous and exogenous ligands. Strong inducers among clinically important drugs are phenytoin and phenobarbital (both used to treat epilepsy), rifampicin (for tuberculosis), and ketoconazole (for fungal infections). All these drugs are also substrates of CYP3A4, as are synthetic steroid

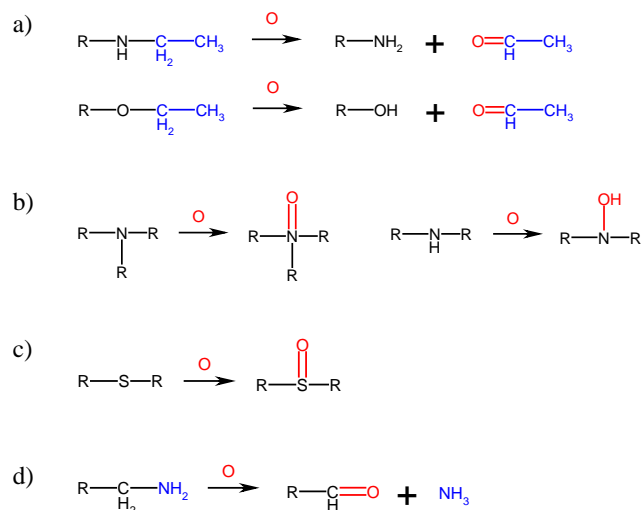


Figure 2.25. Different types of drug modifications catalyzed by cytochrome P450 enzymes. a: N- and O-dealkylation; b: N-oxidation and -hydroxylation; c: sulfoxide formation; d: oxidative deamination.

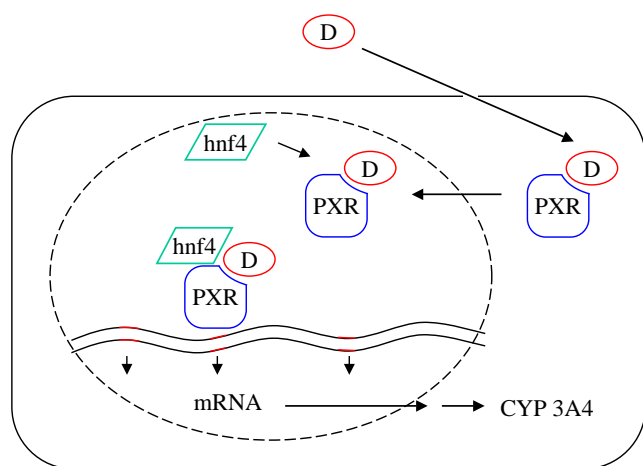


Figure 2.26. Overview of the induction of cytochrome P450 3A4. A drug (D) binds to the pregnane X receptor (PXR); the complex moves to the nucleus, recruits additional proteins (only one of which is shown) and binds to specific regulatory sites on the DNA. This will induce transcription of CYP 3A4 mRNA, as well as other proteins such as conjugating enzymes and membrane transport proteins.

hormones used for contraception. This leads to a variety of clinically important drug interaction phenomena: Oral contraception will cease to work under treatment with rifampicin or phenytoin; dosages of phenytoin will have to be increased during concomitant treatment with rifampicin, etc.

Another, homologous nuclear receptor / transcriptional regulator called AHR (aromatic hydrocarbon receptor) responds to (surprise) aromatic hydrocarbons such as benzo[a]pyrene, and it induces the enzyme cytochrome P450 1A1. This enzyme will not only perform hydroxylations but also introduce an epoxy group into the aromatic system. Polycyclic aromatic hydrocarbons tend to 'intercalate' between the base pairs of DNA, where the epoxy group will react with some amino group, thus covalently fixing the damage in the DNA (Figure 2.27). Although DNA repair mechanisms do exist, they are not 100% effective. Introduction of epoxy groups into initially inert molecules thus converts them into reactive ones that may potentially cause mutations and, ultimately, cancer. This reaction is not at all limited to liver tissue but is ubiquitous; it very commonly occurs in the lungs. In fact, benzo[a]pyrene and related compounds – formed during combustion of tobacco or the allegedly indispensable wonder drug marijuana – are responsible for the induction of lung cancer.

2.4.3. Overview of drug conjugation reactions

We have already seen a variety of conjugation reactions in the foregoing examples. Important reactions are

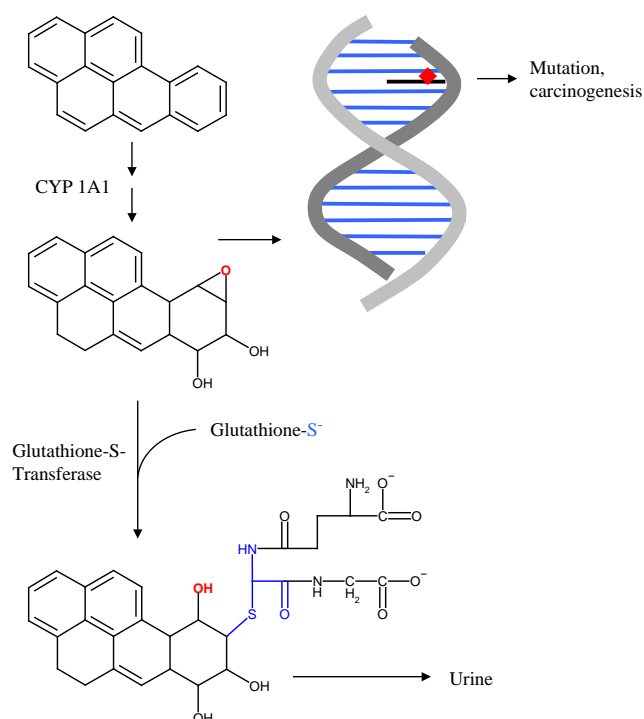


Figure 2.27. Mutagenesis and carcinogenesis by benzo[a]pyrene metabolites. The hydrocarbon, which is inert by itself, is converted by cytochrome P450 1A1 to the 'ultimate carcinogen' 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. This intercalates into and reacts with DNA, which causes mutations and may lead to cancer. The compound may also be conjugated (and thereby detoxified) by conjugation to glutathione.

- **Glucuronidation.** These reactions are catalysed by glucuronosyltransferases and use the cosubstrate UDP-glucuronic acid. The glucuronate is most commonly transferred to a hydroxyl group or to an amino group.
- **Acetylation.** This is mediated by acetyltransferases, uses acetyl-CoA and again mainly involves hydroxyl or amino groups.
- **Sulfation.** Sulfotransferases use 3-phosphoadenosine-5-phosphosulfate (PAPS) as a cosubstrate. It concerns mostly hydroxyl groups.
- **Methylation.** Methyltransferases use S-Adenosylmethionine as cosubstrate. Targets are hydroxyl, amino and sulfhydryl groups.
- **Glutathione conjugation.** This is particularly important with epoxides (Figure 2.27) but may also affect other functional groups.

All the cosubstrates that occur in drug conjugation (Figure 2.28) have other roles in metabolism; e.g., UDP-glucuronic acid and PAPS provide acidic groups for the synthesis of mucopolysaccharides, whereas S-adenosylmethionine provides methyl groups for the synthesis of phosphatidylcholine from phosphatidylethanolamine.

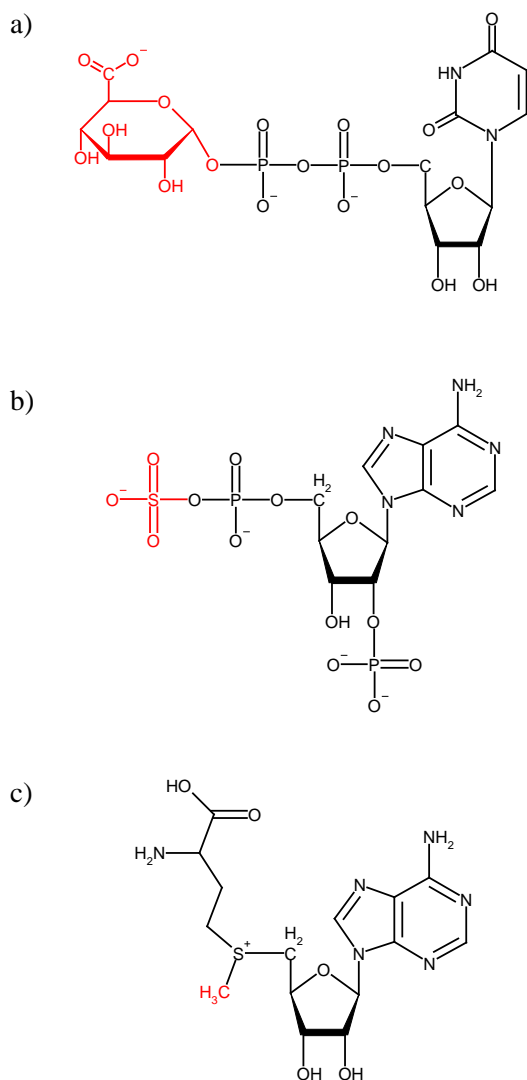


Figure 2.28. Coenzymes that are important in drug metabolism. The groups transferred to the drugs are shown in red. a: UDP-glucuronide (transfers glucuronate), b: PAPS (transfers sulfate), c: S-adenosylmethionine (transfers methyl group).

Glucuronidation, sulfation and glutathione conjugation will all increase the polarity of the drug substrate and therefore facilitate renal elimination. In contrast, methylation would not seem to increase the polarity of the drug. Nor would it render the drug any more amenable to further conjugation; rather, the methyl group introduced would tend to block reactive groups that could otherwise be used for the attachment of glucuronic acid. What, then, is the ‘rationale’ of methylation? It may simply consist in the reduction or abolition of the drug’s specific activity by the change in its structure. The same would seem to apply to acetylation, which utilizes the good, old acetyl-CoA as a cosubstrate³,

³In fact, both methylation and acetylation are very common as mechanisms of bacterial resistance to antibiotics – which suggests that these reactions may have protective functions in mammalian / human drug metabolism, too.

and is shown in Fig. 2.30 for isoniazid (= isonicotinic acid hydrazide), a tuberculostatic agent.

2.4.4. Glucuronidation

Phenolic or alcoholic hydroxyl groups are the most common functional groups to be conjugated with glucuronic acid, as shown above for phenobarbital and morphine. Other possible sites of attachment include carboxylic acids, amines, hydroxylamines, and thiol groups. This versatility is in keeping with the fact that glucuronidation is the most common type of drug conjugation. With this modification, the drug molecule acquires a negative charge and several hydroxyl groups, which will render it considerably more polar and thus fit for excretion. Excretion may happen either by way of the urine, or via the bile⁴. Hepatic secretion (into the bile) works efficiently because all cells in the liver tissue are not only connected to the blood vessels but also to capillary tributaries of the bile duct. Glucuronides may be cleaved in the large intestine by bacteria eager to metabolize the glucuronic acid. One such bacterium that possesses glucuronidase is our good friend *Escherichia coli*. The released drug or metabolite may then be taken up from the intestine again and then reach the liver, thus undergoing a so-called enterohepatic cycle (Fig. 2.22, above). This effect may result in considerably delayed drug elimination. A practically important example is digitoxin (discussed in the chapter on calcium), which is used in the treatment of heart disease. The half-life of this drug extended to several days because of enterohepatic cycling. It is nevertheless often preferred over its analogue digoxin (which is renally eliminated) in those patients who have impaired kidney function.

2.4.5. Glutathione conjugation

Glutathione is involved as a reducing agent in a multiplicity of reactions in cell metabolism. Because of its free sulfhydryl group, it is a very strong nucleophile, and because of that it is useful in the detoxification of the more difficult substrates such as epoxides. Its depletion by drug conjugation may result in severe liver damage. An example of such toxicity is acetaminophen, which at standard dosages is a well-tolerated drug but is highly toxic to the liver at just 3 or 4 times that amount (Figure 2.29).

⁴This latter excretion route is the norm for the endogenous metabolite bilirubin diglucuronide, a degradation product of heme. Bilirubin diglucuronide is modified by bacteria in the large intestine; the conversion products give the feces their characteristic colour. If biliary secretion is blocked (e.g., by a compression of the bile duct), the feces are pale, and bilirubin diglucuronide accumulates in the body, giving the patient a decidedly yellow complexion.

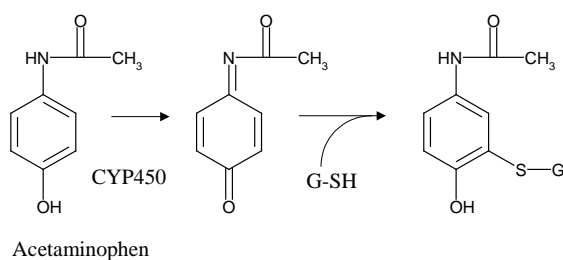


Figure 2.29. One metabolic route of acetaminophen elimination. Initial oxidation (by a cytochrome P450 enzyme) is followed by conjugation to glutathione (G-SH).

2.4.6. Acetylation

The ‘classical’ model of drug metabolism by acetylation is the tuberculostatic drug isoniazide (isonicotinic acid hydrazide). The metabolism of isoniazide has two interesting aspects: Firstly, non-enzymatic hydrolysis of the acetyl metabolite releases acetylhydrazine, which in turn is toxic. This, then, is an example of detrimental drug metabolism (Figure 2.30a, b).

Secondly, the rate of the enzymatic acetylation shows considerable inter-individual variation. This is illustrated in Fig. 2.30c. Shown are the plasma levels of unconjugated (i.e., not yet acetylated) isoniazide in the plasma, 6 hours after intake of a certain dosage of the drug.

The distribution is clearly bimodal (which means, it has two separate peaks). People with a plasma level of more than 2.5 mg/l are deemed ‘slow acetylators’. This is actually a genetic trait that follows Mendelian inheritance, and it is obviously important for the individual adjustment of isoniazide dosage. It is the ‘classical’ but by no means single example of genetic variation in drug metabolism. The study of phenomena of this type is called ‘pharmacogenetics’, and there actually is a scientific journal of that name.

2.4.7. Other reactions in drug metabolism

The conversions of prontosil (azo reduction) and bacampicillin (ester hydrolysis), discussed in the introduction, are examples of other important reactions in drug metabolism.

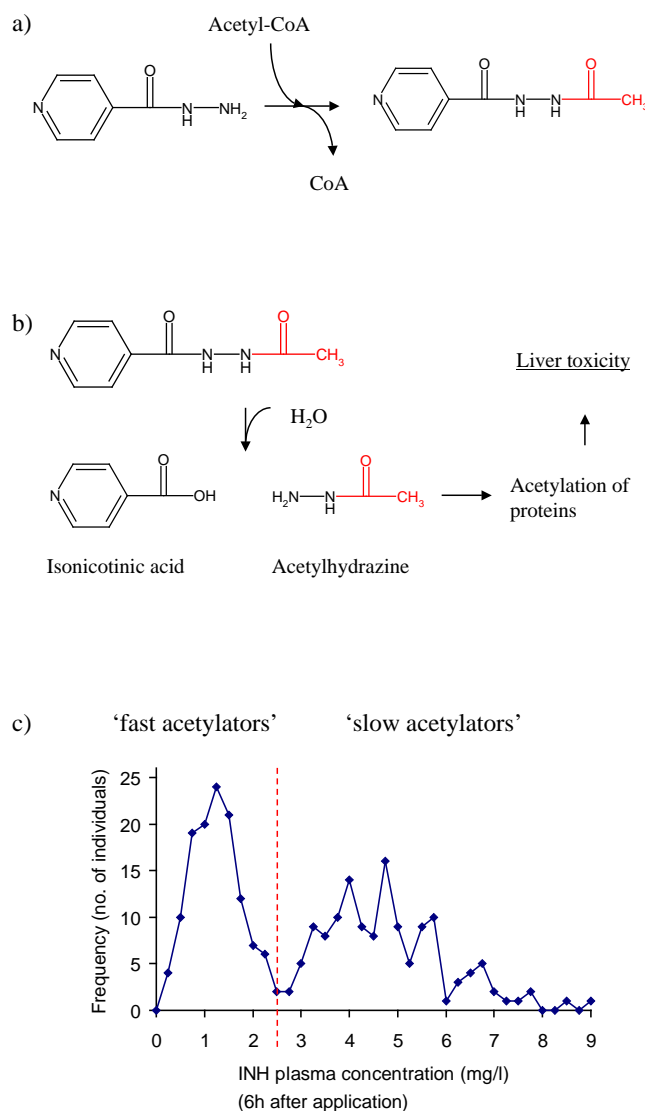


Figure 2.30. Metabolism of isoniazide. a: Acetylation. b: Hydrolysis of the acetyl conjugate leads to liver toxicity. c: Distribution of acetylation rates in the population.

(Notes)

Chapter 3. Pharmacodynamics

Pharmacodynamics starts where pharmacokinetics left off – it assumes that the drug has managed to reach its target, and looks at the principles that govern the interaction between the two.

Almost all drugs will trigger their effects by binding to a receptor. In physiology, the term ‘receptor’ is limited to the sites of action of hormones, neurotransmitters or cytokines. While many drugs do indeed bind to such receptors, in pharmacology the term is used in a more inclusive sense and is applied to other targets such as enzymes and cytoskeletal proteins as well.

3.1. Classes of drug receptors

Drug receptors are mostly proteins. Most of these fall into one of the following categories:

- Enzymes
- Ion channels:
 - Ligand-gated channels: Ion channels that open upon binding of a mediator
 - Voltage-gated channels: Ion channels that are not normally controlled by ligand binding but by changes to the membrane potential
- ‘Metabolic’ receptors – hormone and neurotransmitter receptors that are coupled to biochemical secondary messengers and effector mechanisms. Most metabolic receptors that are drug targets belong to the family of G protein-coupled receptors.
- Cytoskeletal proteins that are involved in cell motility – e.g., actin or tubulin.

Drug target sites that are not proteins include:

- DNA: This is very common with cytotoxic drugs used in cancer therapy, e.g. alkylating agents. These are generally of very poor selectivity and therefore highly toxic. This degree of toxicity is only acceptable in the treatment of life-threatening diseases such as cancer.
- RNA: Although not yet important in clinical practice, antisense oligonucleotides are a very important topic in experimental drug development. These are short, synthetic sequences of DNA (or modified versions of DNA), designed to bind to and inactivate RNA transcribed from specific genes. While this is a theoretically extremely elegant and versatile approach, it has so far

remained largely experimental, despite considerable efforts in the last 10-15 years.

- Membranes: Inhalation anaesthetics (diethylether, chloroform, and their more modern replacements). The mode of action of these was enshrouded in mystery for a long time, but accumulating evidence now supports direct interaction with several ion channels. Nevertheless, there is a remarkably close correlation between the ability of these agents to partition into lipid membranes, as measured by their oil-water partition coefficients, and their narcotic activity; so, in a sense, cell membranes may be considered the targets of these agents.
- Fluid compartments: Osmotically active solutes. These are in fact the only clear exceptions I can think of to the principle that a drug has to bind before acting. Applications include:
 - Plasma volume expanders. If blood is lost during trauma, the loss of volume is more immediately threatening than the loss of red blood cells. Replacement with salt solutions does not work well because small solutes get rapidly filtrated into the interstitial fluid compartment. Only macromolecules are retained in the intravascular space and can prevent filtration of the diluted plasma due to their osmotic activity. Commonly used plasma expanders are metabolically inert polysaccharides such as dextran and hydroxyethyl-starch.
 - Osmotically acting diuretic agents. These are applied in the treatment of intoxication in order to increase the urine volume and accelerate elimination of the poison (‘forced diuresis’). The classical example is mannitol. This sugar is quite similar to glucose in structure but does not get metabolized nor re-absorbed from the primary glomerular filtrate in the kidneys.
 - Laxatives. Example: Sodium sulfate; effective but obsolete.

However, again, most drugs act directly on receptors that are proteins, and for the rest of this chapter we will deal with this major case only.

3.2. Mechanisms and kinetics of drug receptor interaction

There are several typical mechanisms of action that apply to the different types of receptor proteins. For enzymes, these are

- **Competitive inhibition:** The drug occupies the active site and prevents binding of the physiological substrate. Example: The inhibition of angiotensin convertase by enalapril.
- **Irreversible (covalent) inhibition:** The drug again binds to the active site of the enzyme and then covalently reacts with it, so that the active site becomes irreversibly blocked. Example: Inhibition of cyclooxygenase by acetylsalicylic acid.
- **Allosteric inhibition:** The drug binds outside the active site but prevents the enzyme from adopting its active conformation. Example: Inhibition of Na^+/K^+ -ATPase by digitoxin or digoxin.

The allosteric behaviour seen with many enzymes is also typically observed with ion channels and metabolic receptors. In the absence of physiological agonists, these proteins typically prefer their inactive conformation; channels will be closed, and metabolic receptors will not stimulate their downstream cascades. The physiological agonists act allosteric activators, promoting conversion to the active state. Drugs acting on these targets typically belong to one of the following classes:

- **Reversible agonists (activators),** i.e. the drug mimics the physiological agonist. Example: Isoproterenol, an agonist at β -adrenergic receptors.
- **Reversible inhibitors:** The drug, typically in a competitive way, prevents binding of the physiological agonist. Example: Propranolol, an antagonist at β -adrenergic receptors.
- **Reversible partial agonists:** The drug has activity intermediate between that of an inhibitor and an agonist. Example: Dobutamine, a partial agonist at β -adrenergic receptors. Partial agonists may be used for their agonistic properties or their antagonistic properties.
- **Irreversible (covalent) inhibitors.** This case is less common than reversible inhibition or activation. Example: Phenoxybenzamine, an antagonist at α -adrenergic receptors.

With few exceptions, all drugs we are going to consider in the rest of this course will fall into one of the above categories.

3.2.1. Mass action kinetics of drug-receptor binding

In the simplest possible case, one effector molecule, which may be either the physiological agonist or a drug, will bind to one target molecule, and all target molecules will bind the effector with the same affinity. It is noteworthy that there are numerous deviations from this simple situation¹. Nevertheless, we will confine ourselves to this simple model, which will still take us to some important conclusions.

With the above assumptions, the binding will be subject to the law of mass action, and a single parameter – the dissociation constant, typically called K – will describe the interaction. K will be an empirical value, depending on both the ligand and the receptor molecule in question. K is inversely related to the affinity; the higher it is, the lower the binding affinity. The law of mass action can be rearranged to give us the receptor occupancy, i.e. the fraction of all receptors saturated with the ligand (Figure 3.1a). You will recognize the formal similarity to Michaelis-Menten enzyme kinetics. Accordingly, if we plot the receptor occupancy as a function of the ligand concentration, we get the same hyperbolic type of curve (Figure 3.1b, top).

Shown are three curves, differing in their respective values for K . The bottom panel shows that plotting the same numbers on a logarithmic scale for the ligand yields nice sigmoidal plots, which are now distinguished solely by their parallel offsets along the x-axis. From these plots, K can be determined as the ligand concentration of half-maximal receptor occupancy.

If a drug *activates* its receptor, it simply assumes the role of the ligand in the above model, albeit its affinity will most likely differ from that of the physiological ligand. What we can see, then, is that very little benefit can be expected from increasing the drug concentration beyond, say, five times its K value, since the receptor will already be saturated. The only thing that will happen upon further increase is that secondary, less affine and specific sites will be bound, potentially evoking unwanted side effects.

If the drug is an *inhibitor*, we are dealing with a ternary system of receptor, physiological agonist, and our inhibitory drug. We will examine two cases: Reversible competitive inhibitors (Fig. 3.2, top) and irreversible ones (Fig. 3.2, bottom).

3.2.2. Reversible inhibition

If a drug does not undergo a covalent reaction with its receptor, binding will almost always be reversible². There-

¹For example, the nicotinic acetylcholine receptor is known to have two binding sites, which are cooperative, meaning that binding of the second molecule of acetylcholine is more facile.

²One exception is reserpine; it binds non-covalently but with so high

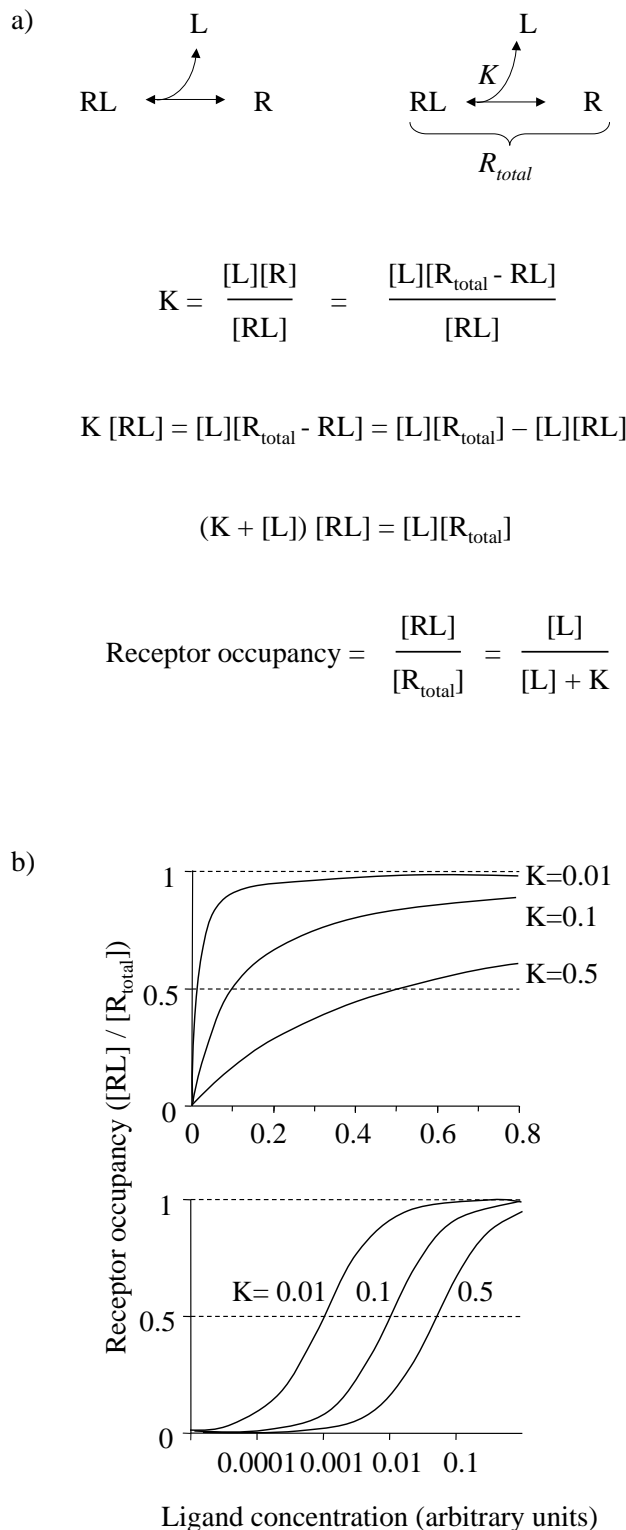


Figure 3.1. a: Derivation of the receptor occupancy function from the law of mass action. This is entirely analogous to Michaelis-Menten enzyme kinetics. b: Linear (top) and semilogarithmic plots of receptor occupancy against ligand concentrations, assuming mass action kinetics. Curves are shown for three different values of K .

affinity that it virtually never leaves the receptor again. There may be others.

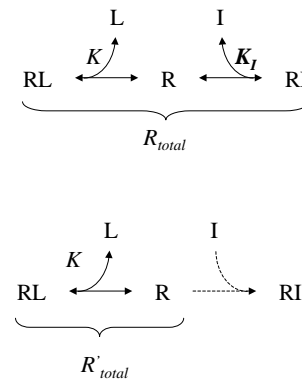


Figure 3.2. Modes of receptor inhibition by ligands. Top: Reversible inhibition. The total pool of receptor molecules is undiminished, but it is now shared by two competing equilibria. Bottom: Irreversible inhibition. Binding and reaction with the inhibitor permanently removes the receptor molecule from the total receptor pool; in the diminished pool, there remains a single equilibrium of ligand binding.

fore, the total number of functional receptor molecules will not change, but we now have two linked, competing equilibria squeezed into the same pool. This gives rise to a modified relationship of receptor occupancy to ligand concentration, as stated and illustrated in Figure 3.3. Again, the situation is entirely analogous to reversible inhibition in Michaelis-Menten kinetics³, and you may want to consult your biochemistry textbook for the derivation – or just do it yourself, as an exercise.

An important aspect of competitive inhibition is that, with sufficiently high concentrations of physiological ligand, the receptor can still be maximally activated. Competitive inhibition thus reduces the receptor's sensitivity to the agonist but does not diminish the maximum effect that can be attained at very high agonist concentrations. This means that, in case of an accidental overdose of the inhibitor, the endogenous agonist or a drug that mimics it could be used to overcome the inhibition.

3.2.3. Irreversible inhibition

If a drug undergoes a covalent reaction with its receptor, the receptor molecules affected will be irreversibly blocked and thus altogether removed from the total receptor pool available for the interaction with the agonist. Thus, the agonist-receptor equilibrium now plays out in that reduced total pool. The number of occupied receptors will therefore be proportionally reduced (Figure 3.4).

³In fact, if R is an enzyme, this actually *is* Michaelis-Menten kinetics.

$$\text{a) } K = \frac{[L][R]}{[RL]} \quad K_i = \frac{[I][R]}{[RI]} \quad [RL] = [R_{\text{total}}] - [RI] - [R]$$

$$\text{Receptor occupancy} = \frac{[RL]}{[R_{\text{total}}]} = \frac{[L]}{[L] + K \left(1 + \frac{[I]}{K_i} \right)} = \frac{[L]}{[L] + K'}$$

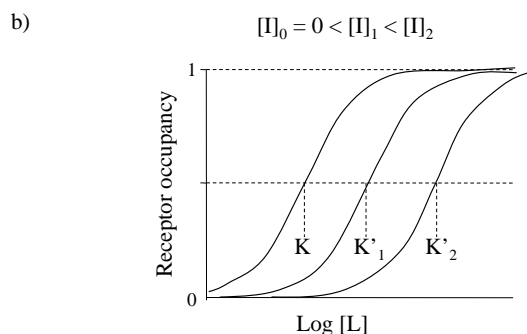


Figure 3.3. Quantitative treatment of competitive inhibition. a: Starting assumptions and derived receptor occupancy as a function of both ligand and inhibitor concentration. At a given fixed inhibitor concentration, the effect of the inhibitor can be described as an increase in the apparent dissociation constant for the agonistic ligand (K'). b: Plots of receptor occupancy vs agonist concentration in the presence of different inhibitor concentrations. The maximal extent of receptor occupancy (and activation) will still be achieved at sufficiently increased ligand concentration.

3.2.4. Example: Inhibition of α -adrenergic receptors by tolazoline and phenoxybenzamine

For an experimental illustration of the foregoing, let us look at the inhibition of α -adrenergic receptors. These receptors are stimulated by epinephrine and norepinephrine; stimulation will increase the tension of blood vessel walls and therefore enhance blood pressure. α -Adrenergic receptors are very numerous in the spleen. The spleen has a sponge-like structure and stores about half a litre of blood, which upon adrenergic stimulation will get squeezed out into the circulation⁴. This extrusion of blood is effected by the contraction of smooth muscle cells that are embedded in the spleen tissue. Accordingly, if we take a fresh slice of spleen and bathe it in solutions of mediators or drugs, we can measure its mechanical tension to quantify the extent of α -adrenergic stimulation. Figure 3.5a shows the force of contraction developed by such spleen strips in response to varying concentrations of norepinephrine, in the presence of tolazoline or phenoxybenzamine, respectively. By com-

$$\text{a) } [R'_{\text{total}}] = [R_{\text{total}}] - [RI]$$

$$\text{Receptor occupancy} = \frac{[RL]}{[R'_{\text{total}}]} = \frac{[L]}{[L] + K} * \left(1 - \frac{[RI]}{[R_{\text{total}}]} \right)$$

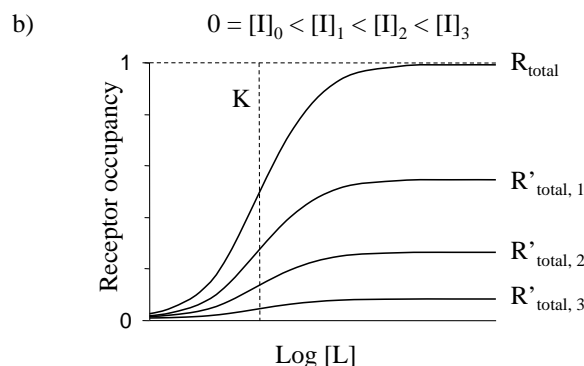


Figure 3.4. Quantitative treatment of irreversible inhibition. a: Starting assumptions and derived receptor occupancy. $[RI]$ will depend on inhibitor concentration but also other things such as speed of reaction and of elimination. We assume that the reaction is complete and $[RI]$ is stable when the ligand binds. b: Plots of receptor occupancy vs agonist (L) concentration after exposure to different inhibitor concentrations. The binding equilibrium (K) for the agonist will be unchanged, but the maximum occupancy will be reduced in a dose-dependent fashion.

parison to the theoretical plots above (Fig. 3.3, 3.4), you will be able to decide which of the two inhibitors is the reversible one, and which is the covalent one.

Let us consider the molecular principles behind the two modes of inhibition. Fig. 3.5b shows the structures of the agonist (norepinephrine) and of the two inhibitors. With some imagination, one can spot the similarity between agonist and inhibitors, so that it is understandable that they all bind to the same site on the α -adrenoceptor. Tolazoline has no obvious reactive groups, and it will therefore bind non-covalently and reversibly.

Phenoxybenzamine, on the other hand, has a chloroethyl group (indicated in red) attached to the nitrogen that is quite reactive. It will undergo the reactions depicted in Figure 3.5c. The initial step results in the formation of an ethylenimine group, which is quite reactive because of the ring tension. In a second step, after binding to the receptor, the ring is opened by some nucleophile, most probably the SH group of a cysteine⁵ that is part of the receptor molecule. In

⁴ A kind of endogenous blood transfusion – remember the roles of epinephrine and norepinephrine as ‘fight-or-flight’ hormones

⁵ A recent paper (J Biol Chem. 276:31279-84; 2001) indicates that in the α_2 receptor it is indeed a cysteine. However, we are here dealing with α_1 receptors, for which I haven’t found any experimental data. I did not check whether that cysteine is conserved.

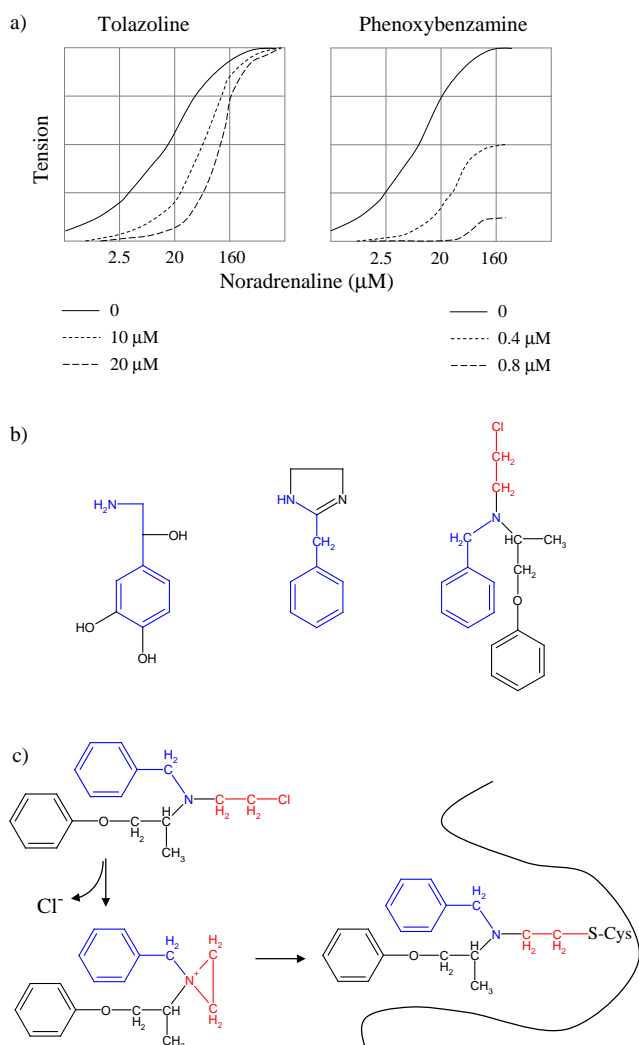


Figure 3.5. Reversible and irreversible inhibition of α -adrenergic receptors in the spleen. a: Contractile tension developed by spleen slices in response to norepinephrine, in the presence of tolazoline and phenoxybenzamine. b: Structures of norepinephrine, tolazoline, and phenoxybenzamine. c: Reaction of phenoxybenzamine with the α -adrenergic receptor. The initial formation of the aziridine ring occurs in solution. The aziridine then reacts with a nucleophilic amino acid side chain (most probably a cysteine) in the binding site of the receptor.

this way, the drug becomes covalently attached to the receptor and permanently inactivates it.

Several things are notable about the action of phenoxybenzamine:

- The initial circularization (formation of the aziridine ring) is rather slow, causing the pharmacological action to lag behind the plasma levels. On the other hand, receptor blockade will persist long after any excess drug has been eliminated. With most drugs that act by non-covalent association with their receptors, plasma levels correlate much more closely with the intensity of drug action.

- While the benzylamino moiety of phenoxybenzamine (blue in Figure 3.5b) targets it to the α -adrenoceptor, the chemical reactivity of the ethyleneimino group is rather non-selective and will cause molecules not bound to the receptor to react in random locations, potentially causing harm including genetic damage. Accordingly, phenoxybenzamine is not the drug of first choice in most clinical indications of α -adrenoceptor blockade.

Phenoxybenzamine is the drug of choice in one particular disease called *phaeochromocytoma*. This is a tumour of the adrenal glands that produces and intermittently releases very large amounts of epinephrine and norepinephrine, causing dangerous spikes in blood pressure. The superior effect of phenoxybenzamine in phaeochromocytoma is a direct consequence of its covalent mode of binding: The inactivated receptor cannot be reactivated by whatever amounts of hormone released (cf. Figure 3.5a). In contrast, reversible inhibition could be overridden in this particular situation.

3.3. Drug dose-effect relationships in biochemical cascades

Above, we noted the similarity of empirical dose-effect relationships with theoretical plots (Figures 3.5a, 3.3, 3.4). This needs to be qualified in two ways:

1. While the theoretical plots modeled receptor saturation, the experiment measured muscle tension.
2. This similarity is by no means perfect.

The two statements are in fact related. In our example, a perfect similarity of theoretical and experimental plots could only be expected if there were a linear relationship between receptor saturation with norepinephrine and muscle contraction. Considering that muscle contraction is triggered quite a bit downstream of receptor activation, there are numerous possible factors that will ‘distort’ this linearity, and in reality no linear relationship will ever be observed if drug target and drug effect are separated by intervening biochemical cascades. It thus turns out that the shape of a dose-effect relationship will depend very much on the functional proximity of the drug receptor molecule and the observed parameter.

If we can directly observe the function of the receptor, which thus at the same time is the effector, there will indeed be a linear relationship between the receptor interaction of a drug and its effect on function, respectively. As examples, we could name:

- Enzymes – observed function: enzyme catalysis;
- Ion channels – observed function: ion conductivity.

On the other hand, very often the observed effect is measured a long way downstream of the drug receptor, as in our example of smooth muscle contraction and α -adrenergic blockers. Other such examples are

- The inhibition of cyclooxygenase by acetylsalicylic acid, which results in a decrease in prostaglandin synthesis, with perceived pain relief as the functional readout;
- The activation or inhibition of nuclear hormone receptors by synthetic androgens, with a readout (way) downstream of transcriptional regulation such as muscle growth or inhibition of sperm production (a highly educational example, isn't it).

In cases like these, there will be numerous possible reasons for deviations from linearity in the dose-effect relationship. The deviations from linearity may of course take any shape; two typical effects are illustrated in Figure 3.6.

A hormone receptor typically triggers a biochemical cascade with multiple steps that need to occur before a functional effect is accomplished. This indirect coupling has surprising consequences for the relationship between the saturation of the receptor and that of the functional effect. We will consider the effect of cascading mediators in a very simple model⁶, containing the following assumptions (Figure 3.7a):

1. The primary agonist (L) binds to the receptor (R) according to the law of mass action.
2. The ligand-bound receptor promotes the formation of a second messenger (M_2), so that the concentration of M_2 is at all times proportional to the receptor occupancy, with a as their ratio. (This condition would be fulfilled if the rate of M_2 's formation were proportional to $[RL]$, and M_2 's decay a first order process.)

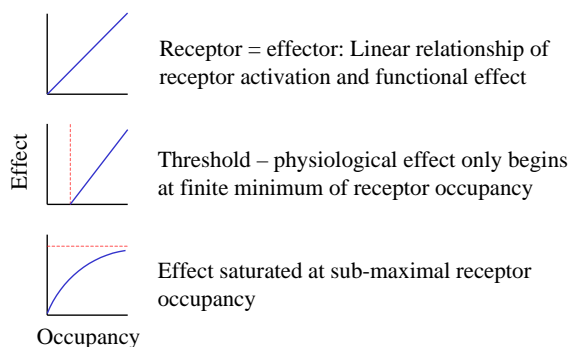


Figure 3.6. Possible relationships between agonist receptor occupancy and corresponding functional effects. A really enlightening figure, isn't it.

⁶Originally described – with some mathematical overhead – by Strickland and Loeb (PNAS 78:1366-70, 1981). Well worth reading.

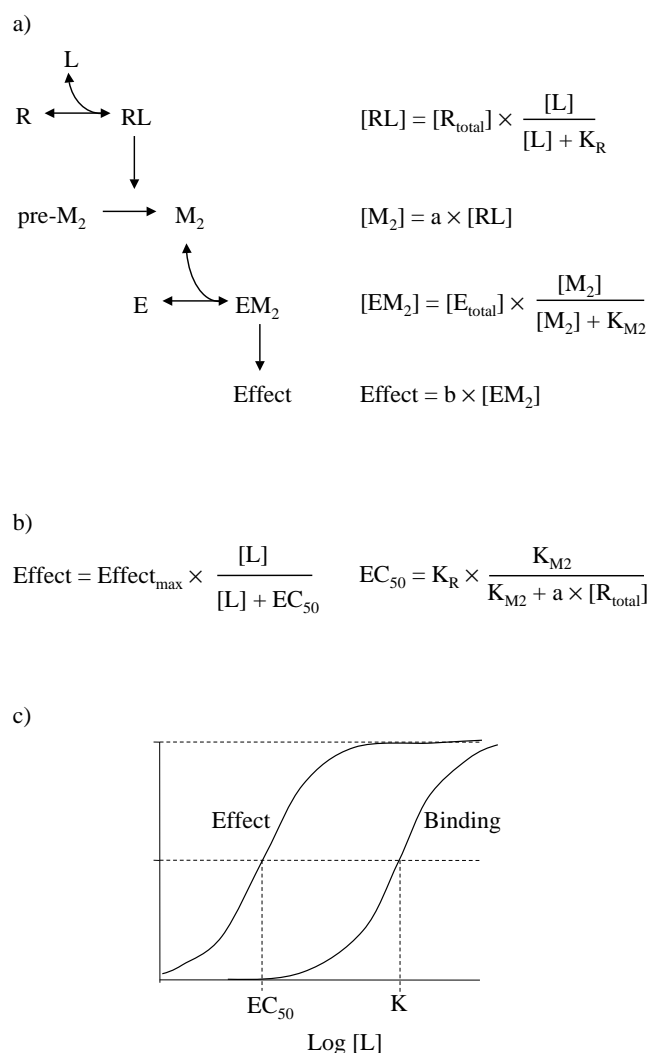


Figure 3.7. Theoretical model to illustrate the effect of biochemical cascades on dose-effect relationships. a: A simple model cascade, containing an agonist (L), a receptor (R), a second messenger (M_2), and an effector (E). b: Equations derived from the assumptions in a. EC_{50} : Ligand concentration required for the half-maximal effect. The effect will saturate at concentrations lower than those required for receptor saturation. The gap between K and EC_{50} depends on the number of receptors and other properties of the system. c: Illustration of the equations stated in b.

3. The second messenger (M_2) saturably binds to the effector, and the observed effect is proportional to the extent of effector saturation with M_2 .

From these fairly straightforward assumptions, the relationships summarized in Figure 3.7b and 3.7c can be derived.

You can see that the relation between effect ligand concentration has the same shape as receptor occupancy. However, the EC_{50} – meaning the ligand concentration required for 50% of the maximum effect – is rather smaller than K_R , the equilibrium constant of receptor binding. Thus, simply because receptor and effector are indirectly connected by means of a second messenger, the functional response

of the system saturates at ligand concentrations that may be substantially lower than those required for saturating the receptor. Importantly, the gap between EC_{50} and K_R will widen with increasing numbers of receptors. Thus, an increase in the number of receptors will always increase the sensitivity of the overall cascade to the ligand, even if all the components downstream of the receptor remain the same.

As an example of this effect, we may consider the stimulation of the heart by epinephrine, which acts on β -adrenergic receptors. Half-maximal increase of heart muscle contractility (i.e., EC_{50}) is observed at 2% receptor saturation. Interestingly, β -receptors are subject to regulation by both covalent modification (phosphorylation) and reversible removal from the cell surface. Either process would reduce the sensitivity of the system to epinephrine but leave the maximum response observed at receptor saturation unchanged.

3.4. Spare receptors

If, as in the above example, some of the receptors can be inactivated without a decrease in the maximal effect, the dispensable receptor fraction is commonly referred to as ‘spare receptors’. Despite its widespread use in the literature, the term is not very precisely defined, and some argument exists about its proper use. Some authors consider a parallel shift of the dose-effect curve in response to an irreversible inhibitor (as for curves 1 and 2 in Figure 3.9) sufficient evidence of spare receptors. Using this interpretation, it would seem that any system with an initial gap between dose-effect and dose-receptor occupancy curves would qualify. Others insist that not only should there be a gap between dose-effect and dose-receptor occupancy curves, but that also the dose-effect curve should be steeper than the dose-receptor occupancy curve.

The second position can be summarized as follows: With or without spare receptors, each occupied receptor molecule should make the same contribution toward the effect until the maximum effect is reached. Therefore, with spare receptors present, 100% of the effect should be reached with less than 100% of the receptors occupied, which means that the response curve for the function must be steeper than that for the receptor occupancy (Figure 3.8). Note that this argument assumes a linear relationship between receptor binding and functional effect. I therefore think that the slope criterion is not generally applicable.

3.5. Potency and efficacy

Two concepts that at this stage should not present us with any difficulty are the ‘potency’ and ‘efficacy’ of a drug. The potency is a function of the amount of drug required for its

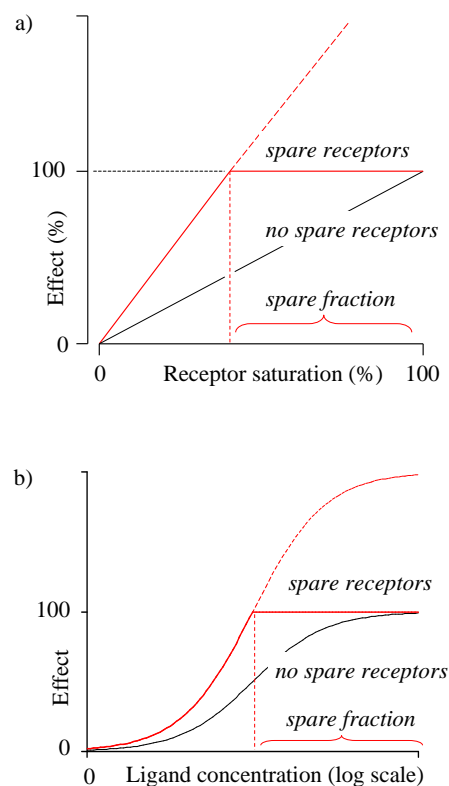


Figure 3.8. The ‘slope criterion’ for spare receptors: Dose-response curves should be steeper for when spare receptors are present. a: Effect vs. receptor saturation. With no spare receptors present, 100% effect occurs only at 100% receptor saturation. With spare receptors, the slope is steeper, and the effect reaches its maximum at less than maximal receptor saturation. The receptors remaining unsaturated at this point constitute the ‘spare fraction’. b: A steeper slope will also manifest in the usual plot (effect vs ligand concentration).

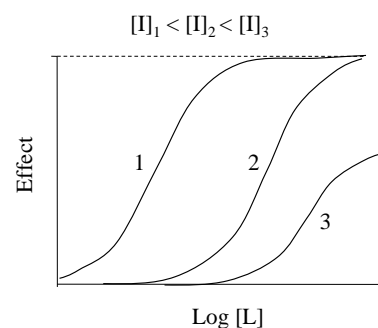


Figure 3.9. Spare receptors and the effect of irreversible inhibitors. The concentration of inhibitor [I] increases from curve 1 to curve 3. Application of $[I]_2$ reduces $[R_{total}]$ but leaves enough receptors to allow for the maximum effect to be regained by increasing the agonist concentration. The eliminated receptors could be considered as ‘spare receptors’. In contrast, $[I]_3$ reduces the receptor concentration below the minimum required for triggering the maximum effect.

specific effect to occur; it is measured simply as the inverse of the EC_{50} for that drug. In contrast, the efficacy measures

the maximum strength of the effect itself, at saturating drug concentrations. Thus, in Figure 3.10, drug Red™ exceeds drug Black® in potency, while the opposite is true of the efficacy.

3.6. Partial agonism and the two-state model of receptor activation

The efficacy will obviously vary for drugs that act on the same physiological parameter by different routes; e.g., morphine is a stronger painkiller than aspirin is. However, profound differences may even be observed with substances that act on the very same site of the very same target molecule. Figure 3.11 shows an example. The receptor in question is a serotonin receptor (subtype 1A) which occurs in the brain and is the target of some psychoactive drugs. Like the adrenergic receptors mentioned above, it is a G protein-coupled receptor. Receptor activation will trigger exchange of GDP for GTP in the cognate G proteins. It can therefore be measured by way of incorporation of $\text{GTP-}\gamma^{35}\text{S}$, which is both radioactive and resistant to the intrinsic GTPase activity of the G protein. You can see that the effects of the different agonists applied not only arise at different concentrations but also level off at different maxima, some of them well below the reference value (100%).

Agonists that show sub-maximal activation of a receptor, even at saturating concentrations, are called ‘partial agonists’. How can an agonist be ‘partial’?

A plausible explanation can be given if we consider the allosteric nature of the ‘typical’ receptor protein and its interaction with the ligand⁷. An allosteric protein has two conformational states that are in equilibrium (Figure 3.12). With most receptors, the inactive state will be more prevalent in the absence of agonists. However, an agonist will exclusively bind to and therefore favour the active confor-

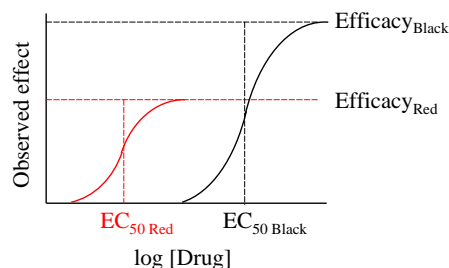


Figure 3.10. Potency and efficacy of a drug. The potency is defined as $1/\text{EC}_{50}$, whereas the efficacy the effect observed at saturating concentrations.

⁷The term ‘allosteric’ is used here in a very elementary sense, meaning that the effector binds in one place and triggers a conformational and functional change to another. It does not imply oligomeric structure of the receptor or cooperativity of binding.

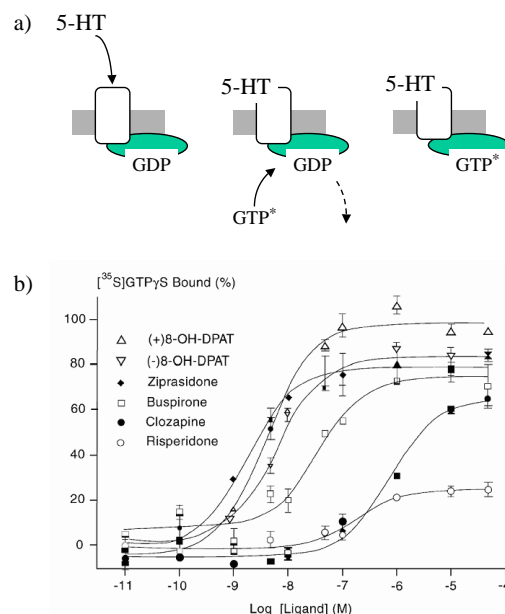


Figure 3.11. Activation 5-hydroxytryptamine (=serotonin) receptors by various agonists. a: Principle of the assay. Cell membranes carrying the receptors and associated G-proteins were incubated with the agonists and with ^{35}S -GTP. The radioactive label will bind to the G protein when the receptor is activated by an agonist. b: Dose-response curves for receptor activation by ^{35}S -GTP binding in response to various agonists.

mation. If the concentration of agonist is sufficiently high, the entire receptor population will be arrested in the active state.

Analogously, an antagonist will preferentially bind the inactive conformation and therefore, at saturating concentrations, convert the entire receptor population to the inactive state. A *partial* agonist will have finite affinity for both conformational states of the receptor, although it will be higher for the active conformation, so as to overcome the intrinsic preference of the inactive state (K_{intr} in Figure 3.12) and therefore bring about any appreciable receptor activation at all. Differences in the maximum effect (or efficacy) between different partial agonists then simply correspond to different ratios of K_A/K_P , as defined in Figure 3.12.

As you can see in Figure 3.12 (bottom row), the receptor assumes four distinct states:

$$[R_{\text{total}}] = [R_{\text{active}}] + [R_{\text{active}}L] + [R_{\text{inactive}}] + [R_{\text{inactive}}L]$$

The active fraction of the receptor comprises the free and the ligand-associated active forms:

$$\text{Active fraction} = ([R_{\text{active}}] + [R_{\text{active}}L]) / [R_{\text{total}}]$$

The expression that quantitatively describes the active fraction as a function of the ligand concentration can quite easily be derived using the equilibrium equations, together with the two equations above:

$$K_A = [R_{active}][L] / [R_{active}L]$$

$$K_I = [R_{inactive}][L] / [R_{inactive}L]$$

$$K_{intr} = [R_{active}] / [R_{inactive}]$$

It works out to (fasten your seatbelts):

Active fraction =

$$(K_A + [L]) / ((K_A + [L])(1 + 1/K_{intr}) + [L](K_A - K_B) / (K_{intr} K_B))$$

I will post an Excel spreadsheet on the course website that will allow you to play a bit with the parameters and see how they affect the resulting curve.

Are partial agonists clinically relevant? Once upon a time, partial agonists for β -adrenergic receptors were promoted as a ‘milder’ alternative to β -blockers, but this seems to have been abandoned. A practically more interesting application are synthetic opioids. These are being used for analgesia, i.e. as pain killers. While morphine or other full agonists such as fentanyl are the strongest pain killers available, partial agonists such as nalorphine or pentazocine are

being used in applications requiring something intermediate in strength between morphine and basic pain killers such as aspirin. The rationale for preferring partial agonists over the ‘real thing’ is that the partial agonists also seem to have a lower addictive effect than the full agonists, although this is not undisputed⁸.

3.7. Toxic and beneficial drug effects

As an anticlimactic finale to this theory-heavy topic, let us consider the relationship between beneficial (therapeutic) and toxic (side) effects of drugs. Possible forms of this relationship are schematically depicted in Figure 3.13.

The first case applies if toxicity arises simply as an extension of the therapeutic effect. As an example, consider warfarin, an inhibitor of an enzyme necessary in the post-translational modification of blood coagulation factors⁹. While it may help to prevent thrombosis and stroke when used in low amounts, any excess in drug effect will be highly dangerous, leading to things such as spontaneous hemorrhage into the brain.

Toxicity as an extension of therapeutic action is usually associated with a small *therapeutic index*, which is simply the ratio of the toxic plasma concentration over the therapeutic plasma concentration. It should be apparent that drugs with a small therapeutic index require the most attention and alertness with respect to variations in metabolism and elimination. Such variations may easily cause the concentration within the body to either exceed the toxicity threshold, or drop below the minimum amount required for the therapeutic effect. Accordingly, in our example, patients receiving warfarin treatment need to have their blood clotting function measured at regular, frequent intervals.

In the second case in Figure 3.13, the therapeutic and the toxic effects are triggered from different receptors altogether. The challenge then will be to find drugs that will selectively act on the receptor responsible for the therapeutic action. As an example, we may cite β -adrenergic receptors. The blockade of β receptors in the heart is used in the treatment of hypertension and of heart disease; on the other hand, blockade of β receptors in the bronchi will promote bronchoconstriction and may aggravate the symptoms of asthma. The β receptors in the heart mostly belong to the β_1 subtype, whereas in the bronchi we mainly find β_2 receptors. β_1 -Selective adrenergic antagonists (‘cardioselective

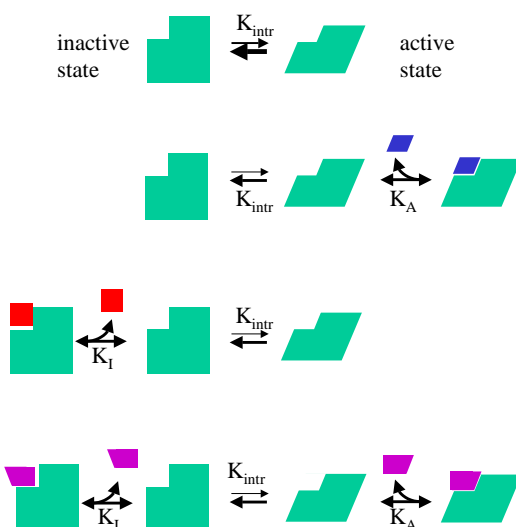


Figure 3.12. The allosteric model of receptor activation, inhibition and partial agonism. The receptor itself is characterized by an intrinsic conformational equilibrium (K_{intr}) that favours the inactive state. Agonists bind exclusively to the active state, with a characteristic dissociation constant K_A and thus increase the overall amount of active receptor molecules. Inhibitors analogously prefer the inactive conformation, whereas partial agonists can bind either conformation and thus will cause a fraction of the receptor to remain in the inactive conformation.

⁸The issue is complicated by the fact that, with opioid receptors, there are again multiple subtypes, and the efficacy and potency of each drug may be different for each of them.

⁹ It is actually an antimetabolite of vitamin K. Voet&Voet have all the details.

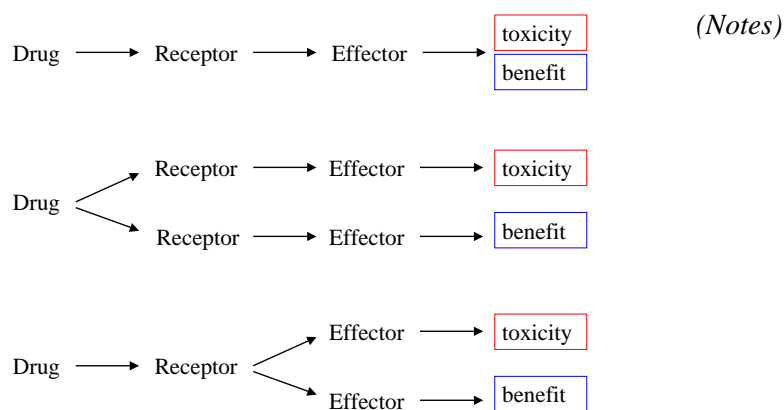


Figure 3.13. Different relationships of toxicity and benefit associated with drug action.

β blockers') are available and preferably used in asthma patients.

In the third case Figure 3.13, the drug binds to a single receptor alright, but the causal chains leading to the therapeutic and toxic effects, respectively, separate at a point upstream of the ultimate effectors. Inhibitors of monoamine oxidase may serve as an example. Monoamine oxidase (MAO) is responsible for the degradation of epinephrine and norepinephrine, dopamine, serotonin, and histamine. Inhibition of this enzyme will therefore have a broad range of effects. To find better therapeutic options, it would not seem useful to find 'better inhibitors¹⁰', but instead to try and find drugs acting downstream of the branching point. In fact, MAO inhibitors are now obsolete and have been superseded by drugs that act selectively on the receptors for the different hormones.

¹⁰Although there is MAO 1 and MAO 2, and some interest still exists in MAO 2-selective inhibitors.

Chapter 4. The ionic basis of cell excitation

Excitable cells – nerve cells and the various types of muscle cells – have a prominent role in the physiological processes that are targeted by drug therapy. We will therefore spend some time looking at how electrical cell excitation works.

The fundamental prerequisite of excitability is the presence of a membrane potential. A membrane potential is present in apparently all living cells. In non-excitable cells, the orientation of the membrane potential is always such that the cell interior is electrically negative against the outside. This orientation also prevails in excitable cells that are not currently excited, i.e. currently are at their *resting potential*. One fundamental function of this negative-inside membrane potential in all cells consists in powering active transport, usually in the form of sodium cotransport.

Membrane potentials also exist across membranes within cells. An important example is the potential across the inner mitochondrial membrane, which is the major driving force of ATP synthesis. However, since the intracellular potentials don't have a prominent role in cell excitation and pharmacology, the following discussion will focus on the potentials that occur at the cytoplasmic membrane.

In excitable cells, the orientation of the membrane potential is reversed for a brief period of time during excitation. This transient reversal is called the *action potential*. Its duration may vary from ~1 millisecond to several hundred milliseconds, depending on the cell type. An action potential is typically triggered locally on a small patch of the cell membrane. However, from there, it will rapidly spread all over the entire cell membrane, rapidly altering the functional state of the cell. Moreover, excitation of one cell often triggers excitation of neighbouring cells by means of electrical or chemical coupling. The physiological significance of electrical cell excitation thus is that it provides the most rapid means of signal transduction and communication within and between cells.

4.1. Ion gradients across the cell plasma membrane

All membrane potentials depend on the existence of ion gradients across the membrane in question. The major ion species that shape the form of both resting potentials and action potentials are K^+ , Na^+ , Ca^{++} , and Cl^- . The ion gradients result from the activities of three types of membrane proteins:

1. Ion pumps. These proteins use metabolic energy in the form of ATP to transport ions against their concentra-

tion gradients. Quantitatively the most important ion pump is Na^+/K^+ -ATPase (Figure 4.1), which transports both sodium and potassium against their respective gradients (table 4.1). In addition, various types of calcium pumps are found in the cytoplasmic, ER and mitochondrial membranes; the direction of Ca^{++} transport is always from the cytosol to the other compartment.

2. Exchange- and co-transporters. These link the gradients of different ion species to one another, so that gradients can be established for ions for which specific pumps do not exist (or have insufficient capacity). Important examples are the sodium/calcium exchanger and the potassium/chloride co-transporter in the cytoplasmic membrane (Figure 4.1).
3. Ion channels. These proteins simply facilitate the diffusion of ions downhill their concentration gradients, i.e. they tend to dissipate the concentration gradients established by the transporters.

Most, but not all channels can switch between open and closed states. Switching can be accomplished by a ligand binding to the channel or by changes in the surrounding electrical field. Therefore, we have the following major functional groups of ion channels:

1. Ligand-gated channels, which may either open or close in response to ligand binding. Important examples are the nicotinic acetylcholine receptor, which allows Na^+ into the cell in response to acetylcholine, and the sulfonylurea receptor-associated K_{ir} channel, which ceases to permit efflux of K^+ in response to ATP.
2. Voltage-gated channels. The voltage-gated channels for K^+ , Na^+ and Ca^{++} are all involved in cell excitability.
3. 'Leak' channels, which seem to be fairly simple-minded and just continuously permit flux of the cognate ion. The most important ones are those for K^+ ; they are responsible for the fact that K^+ permeability dominates the resting potential.

The continuous flux of ions through leak channels (and exchange transporters) requires continuous operation of the ion pumps. Therefore, a sizeable fraction of our metabolic energy is expended just to keep up the ion gradients across our cell membranes.

The major ion transport processes that are responsible for maintaining the ion gradients and the resting potential across the cytoplasmic membrane are summarized in Figure 4.1:

| Ion species | Concentration inside cell | Concentration outside cell |
|------------------|---------------------------|----------------------------|
| Na^+ | 15 mM | 150 mM |
| K^+ | 150 mM | 6 mM |
| Cl^- | 9 mM | 125 mM |
| Ca^{++} | 100 nM | 1.2 mM |

Table 4.1. Intra- and extracellular ion concentrations

1. Na^+/K^+ -ATPase (top) exchanges 3 Na^+ with 2 K^+ ions for each molecule of ATP consumed.
2. Ca^{++} is transported in exchange for Na^+ , so that the Ca^{++} gradient depends in part on the Na^+ gradient. In addition, Ca^{++} is also extruded by specific pumps (not shown).
3. Chloride is expelled from the cytosol by co-transport with K^+ . The chloride gradient therefore is dependent on¹and sustained by the K^+ gradient.
4. The leak channels cause the permeability of K^+ to be higher than for any other ion species, so that the resting potential is kept close to the K^+ equilibrium potential (see below).

Note that the extra Na^+ ion that is extruded by Na^+/K^+ -ATPase is *not* the immediate cause of the negative-inside membrane potential. Instead, extra sodium ions are allowed back in during active transport of metabolites such as amino acids and (with some cells) glucose, and in exchange for Ca^{++} ; any remaining intracellular cation deficit would be balanced by K^+ flowing back in across its own channels.

4.2. The physics of membrane potentials

Both the resting and the action potential are diffusion potentials. A diffusion potential arises at a membrane if

1. the membrane is selectively permeable for one or a few ion species,
2. Is exist will therefore have a say in determining the membrane potential. Furthermore, as ion channels open and close, the changing permeabilities can shift the weight from one ion to the other. The most important example is the transient opening of sodium channels, which according to the Goldman equation will cause the membrane potential to shift toward the equilibrium potential.

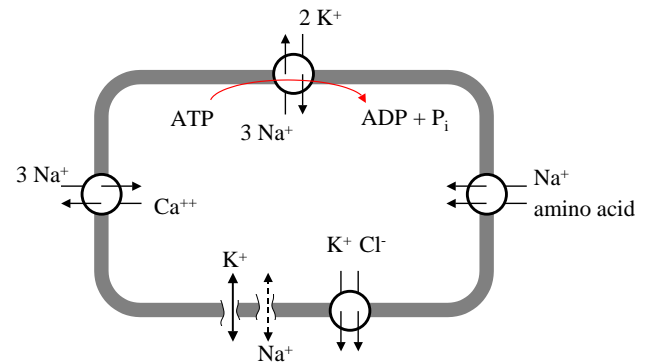
**Figure 4.1.** Major membrane ion transport systems that shape the ion concentration gradients across the cytoplasmic membrane. Top: Na^+/K^+ -ATPase; left: $\text{Na}^+/\text{Ca}^{++}$ -exchanger; bottom: Ion channels for K^+ and Na^+ , and K^+/Cl^- -cotransporter; right: Na^+ -amino acid cotransporter.

Figure 4.2a shows a membrane that has an ion gradient across it but is entirely impermeable; this will result in no membrane potential, as long as the numbers of anions and cations are the same within each adjoining compartment. In Figure 4.2b, the membrane has been rendered permeable by a large, non-selective hole; both anions and cations are free to equilibrate across the membrane – again, there will be no membrane potential. In Figure 4.2c, the membrane contains channels that are selectively permeable to the cations. Diffusion of cations down their concentration gradient will create an imbalance of charge that in turn gives rise to a potential (a voltage gradient) across the membrane.

The migrating cations depicted in Figure 4.2c are subject to two driving forces that act in opposite directions: The concentration gradient will tend to drive any permeable species, regardless of its charge, across the membrane, until the concentrations are identical on both sides (Figure 4.4a); this is simply the maximization of entropy. Conversely, with progressing diffusion, the membrane potential will rise ever higher and tend to pull the cations back to the left side (Figure 4.4b). At some point, the two forces will be equally strong, and no further net ion movement will occur. This point is the *equilibrium potential*.

The equilibrium potential can be calculated from the Nernst equation, which is simply an application of the Gibbs equation to ion gradients (Figure 4.4c)².

You will note that most terms in that equation are constants (the temperature is virtually constant at $37^\circ\text{C} = 310$

¹Table 1 tells us why this works – can you see it?

²The symbols in this equation have the following meanings: ΔE , the diffusion potential; R , the gas constant ($8.31 \text{ J} \times \text{K}^{-1} \times \text{mol}^{-1}$); T , the absolute temperature; F , Faraday's constant ($96500 \text{ Coulomb/mol}$); z , number of charges per ion (1 with K^+ and Na^+ , 2 with Ca^{++} , -1 with Cl^-); \ln , natural logarithm (base: $e = 2.71828$); C_{in} , C_{out} , inside and outside concentrations of the diffusible ion species.

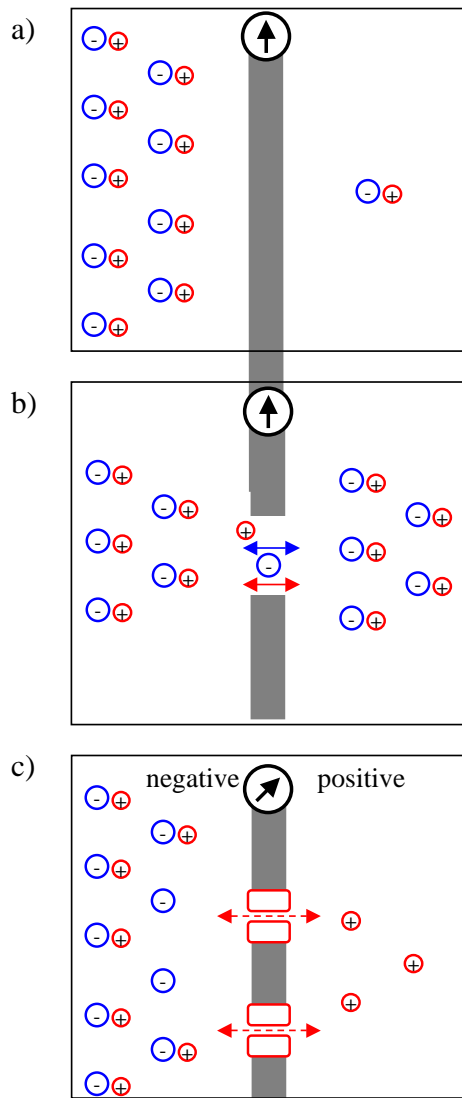


Figure 4.2. The origin of diffusion potentials. An ion gradient across an impermeable membrane will not generate any diffusion potential (a), nor will there be a potential across a membrane that allows both anions and cations to equilibrate (b). However, with selective permeability for one ion species (here, the cations), an ion gradient will create a diffusion potential.

$$\Delta E = \frac{R \times T}{F} \times \ln \frac{P_K \times C_{K,out} + P_{Na} \times C_{Na,out}}{P_K \times C_{K,in} + P_{Na} \times C_{Na,in}}$$

Figure 4.3. The Goldman equation.

K, too), so that the potential is essentially a function of the ion concentration gradient alone. However, this simple relationship will hold only when there is only one diffusible ion species. In a cell, we have several ion species, and finite permeabilities for several of them. The two dominant cations, Na^+ and K^+ , have roughly opposite distributions across the plasma membrane. Application of the Nernst equation to Na^+ and K^+ would yield membrane potentials of +60 mV and -90 mV, respectively³. Yet, the actual resting

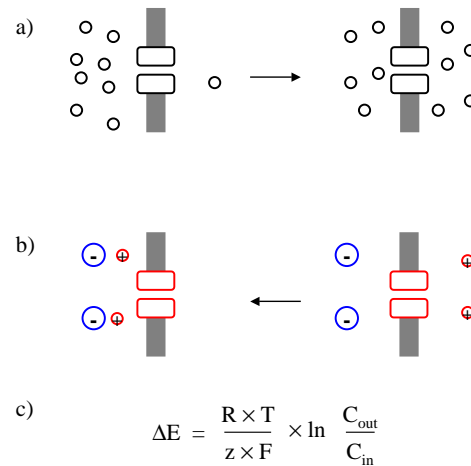


Figure 4.4. Driving forces in ion diffusion across selectively permeable membranes. a: Entropy promotes diffusion down a concentration gradient, regardless of charge. b: Electroneutrality will oppose entropy. c: The Nernst equation describes the membrane potential that ensues when entropy and electroneutrality are in equilibrium.

membrane potential is not identical to either of these values (which it would if the membrane were selectively permeable to either K^+ or Na^+ only). Clearly, what we need is a means to determine the membrane potential if we have multiple permeable ion species. This is provided by the Goldman equation (given in Figure 4.3 in its special case for K^+ and Na^+). When comparing it with the Nernst equation, you see that the Goldman equation introduces one additional parameter P (for permeability), which has a specific value for each ion present.

Thus, the contribution of each ion to the overall membrane potential depends not only on its concentration but it is weighted for the permeability of the membrane for that ion. Since the concentrations and gradients for K^+ and Na^+ are of similar magnitude, we may infer that the membrane permeability must be larger for potassium than for sodium ($P_K > P_{Na}$), since the actual membrane potential is much closer to the K^+ than to the Na^+ equilibrium potential.

Now what's the molecular basis of different permeabilities for different ions? This is where the channels come in. Without a specific channel, no ion can effectively cross the membrane, so its permeability will be very small; only ions for which specific channels exist will therefore have a say in determining the membrane potential. Furthermore, as ion channels open and close, the changing permeabilities can shift the weight from one ion to the other. The most important example is the transient opening of sodium channels, which according to the Goldman equation will cause the

³By convention, a minus sign indicates that the cell interior is negative with respect to the exterior.

membrane potential to shift toward the sodium equilibrium potential.

4.3. Voltage-gated cation channels and the action potential

While transporters and leak channels shape the resting potential, the action potential is the fiefdom of the gated channels. Voltage-gated channels, in particular, are important for the spreading of action potentials over the surface of an entire cell. In most excitable cells, there are high numbers of voltage-gated channels for both sodium and potassium (and, in fact, several subtypes of both). In addition, voltage-gated Ca^{++} channels prominently occur in heart and smooth muscle cells.

In the resting state, the voltage-gated channels are closed. As stated before, the negative-inside potential at rest is due to the occurrence of K^+ leak channels (Figure 4.5a). The voltage-gated channels enter into the picture when a change occurs in the surrounding electrical field. The channel proteins possess mechanically flexible domains that carry an electrical charge, and that will thus change conformation in response to Coulomb forces. If the electrical field is reversed artificially using microelectrodes, the gate of the sodium channel ‘swings’ open (Figure 4.5b), and all of a sudden the permeability of the cell membrane for sodium will exceed that for potassium. According to the Goldman equation, this will lead to the re-orientation of the membrane potential, because the distribution of Na^+ ions is opposite to that of K^+ . This reversal of the membrane is called depolarization. Therefore, the membrane responds to a limited extrinsic depolarization with a much more vigorous depolarization of its own. This is what constitutes membrane excitation.

A crucial aspect of this excitation is that it will spread along the entire expanse of the membrane. The initial, triggering depolarization is usually a localized event; the opening of the sodium channels close by then provides the trigger for channel opening and depolarization in the adjoining regions in turn. This self-sustained, spreading depolarization is called the action potential (Figure 4.5c).

Although the simplified cartoons suggest otherwise, the membrane potential does not actually have to be reversed for the sodium channels to open. As soon as the externally triggered depolarization reaches a threshold value of about -55mV (Figure 4.6b), the sodium channels will start to open, and the action potential will be triggered, causing the depolarization to spontaneously go up to about $+50\text{--}60\text{ mV}$ (Figure 4.6a).

Figure 4.6a shows the trace of an action potential traveling down a nerve fiber, recorded at some distance downstream of the stimulating electrode. It consists of a brief depo-

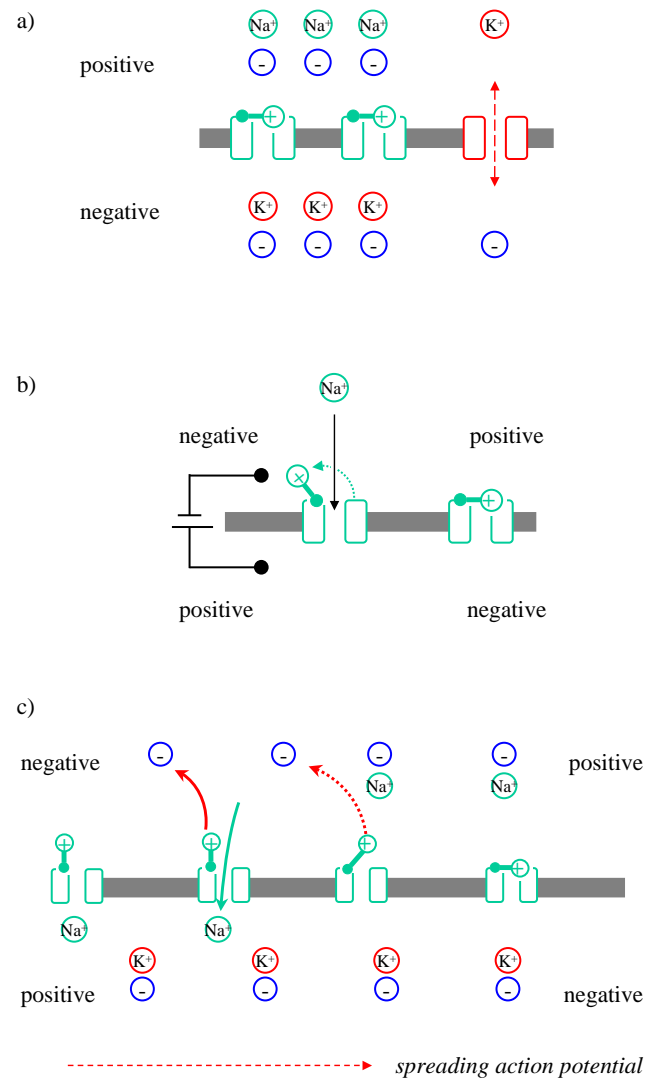


Figure 4.5. Ion channels and the formation of the action potential. In each panel, the lower compartment is the cell interior. a: Na^+ and K^+ permeability of the cytoplasmic membrane under resting conditions (simplified). The voltage-gated Na^+ channels are closed, whereas the K^+ ‘leak’ channels are open. A diffusion potential only exists for K^+ , rendering the interior is electrically negative. b: Opening of a voltage-gated Na^+ channel in response to an externally triggered depolarization (=reversal of the potential). c: Propagation of the action potential by successive sodium channel opening events.

larization spike (duration $\sim 1\text{--}3\text{ msec}$) that approaches but does not quite reach the Na^+ equilibrium potential, followed by a slightly longer lasting depression below the level of the resting potential. Altogether, we can distinguish four phases in the potential curve (see numbers in Figure 4.6a), which can be explained as follows:

1. Phase 1 is the resting potential, maintained close to the K^+ equilibrium potential by the K^+ leak channels.
2. The vigorous rise of the action potential is caused by the opening of voltage-gated Na^+ channels. With the Na^+ permeability now exceeding the K^+ permeability, the

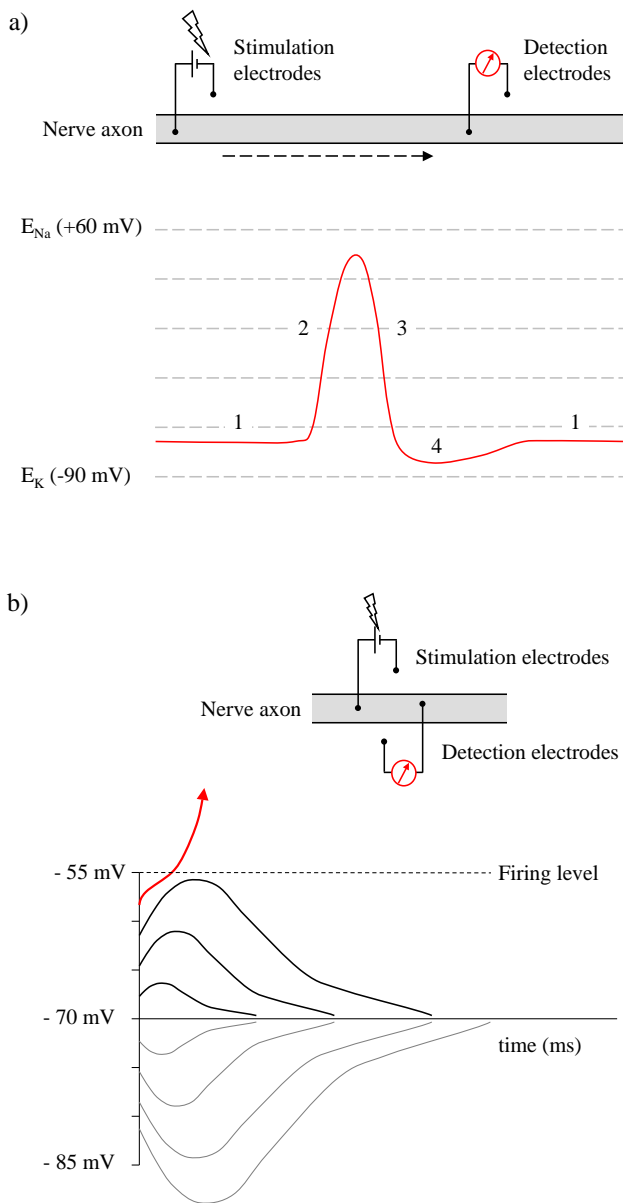


Figure 4.6. Experimental evocation and detection of action potentials. a: Detection of action potential propagation. Top: Schematic of the experimental setup to trigger and detect action potential. Below: Trace of an action potential passing the detection electrodes. The numbers refer to phases 1-4 as explained in the text b: Local stimulation, and the firing level. If a patch of nerve cell membrane is partially depolarized to a level of ~ 55 mV, Na^+ channels will start to open, and an all-out action potential will be triggered. In contrast, depolarizing stimuli that remain below the firing level will just dissipate, as will hyperpolarizing stimuli.

membrane potential is shifted toward the Na^+ equilibrium potential.

3. The equally rapid decline is due to the Na^+ channels beginning to close, and voltage-gated K^+ channels beginning to open. The combined permeabilities of the voltage-gated and the leak K^+ channels exceed the de-

clining Na^+ permeability and pull the potential back toward the K^+ equilibrium potential.

4. The transient hyperpolarization is due to open voltage-gated K^+ channels lingering after the Na^+ channels have already closed.

Like the sodium channels, the voltage-gated potassium channels will finally inactivate. This will revert the membrane potential back to normal.

Thus, two effects are responsible for the limited duration of the action potential (essential if we don't want to throw away the brain cell after single use, kind of wasteful):

1. The inactivation of the sodium channels, which after having stayed open for just a few milliseconds will close despite persisting membrane depolarization;
2. The opening of the voltage-gated potassium channels, which will pull the membrane potential back into the direction of the K^+ equilibrium potential.

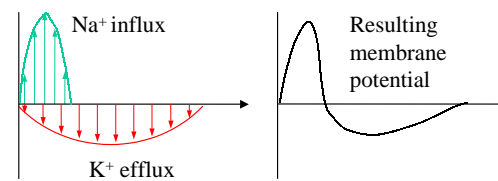


Figure 4.7. Na^+ and K^+ fluxes during the action potential.

While the workings of the sodium and the potassium channels are closely similar, the sodium channels are faster at both opening and closing. This gives the action potential its shape (Figure 4.7).

If depolarization is responsible for opening the channel gate, it can not easily be blamed for its closing (cf. Figure 4.5b). To account for the closing event, we must therefore postulate a separate mechanism (Figure 4.8). The structure of the voltage-gated potassium channel is more or less known (Figure 4.9), and the gate mechanisms for both opening and inactivation can actually be located.

The channel consists of four protein subunits, each of which contributes 6 helices to transmembrane part of the channel. In addition, a sizeable chunk of protein protrudes into the cytosol. The trigger of the opening gate is located on one of the 6 transmembrane helices, which carries several positive charges (an unusual feature for transmembrane helices, which typically have no charges at all). The charge will cause the helix to move when the electrical field in its vicinity is changed, i.e. when the membrane is depolarized. This movement will cause adjacent helices to move in turn and thus open the gate (Figure 4.9b).

The second gate, which is responsible for inactivation, conforms very much to the ball-and-chain mechanism de-

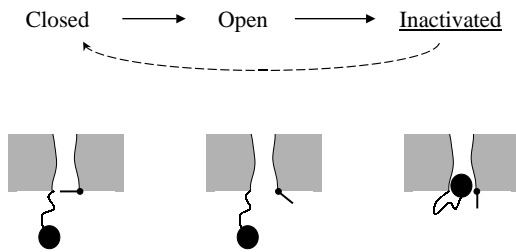


Figure 4.8. The two gate-model of ion channel function. Depolarization flings open the first gate; the channel is subsequently closed by a separate mechanism that inactivates the channel during continuing depolarization. It is noteworthy that this ‘ball and chain’ model had been proposed long before structural details of the channel structure became available.

scribed in Figure 4.8. It consists in a positively charged, flexibly attached N-terminal domain, which likewise undergoes a movement in the changed electrical field and simply plugs the pathway that is transiently opened for the K^+ ions (Figure 4.9c).

4.4. The origin of cell excitation

The mechanisms we have discussed above account for the propagation and for the termination of the action potential. However, so far we have relied on external electrodes for its initiation. Under physiological conditions, action potentials can be evoked in various ways.

The first, very important means of action potential generation consists in synaptic transmission. A synapse connects a presynaptic cell (always a neuron) to a postsynaptic cell (a neuron or muscle cell). In brief, a synapse works as follows:

1. Excitation of the presynaptic cell leads to the release of a neurotransmitter substance.
2. The neurotransmitter binds to a receptor on the postsynaptic cell, very commonly a ligand-gated channel.
3. The receptor channel opens and locally depolarizes the membrane.
4. The local depolarization is picked up by adjacent voltage-gated channels and propagated across the entire membrane of the postsynaptic cell.

A very widespread receptor channel is the nicotinic acetylcholine receptor, which is found on all skeletal muscle cells. Upon binding of the transmitter (acetylcholine), this channel opens up to both K^+ and Na^+ . This would drag the membrane potential towards the mean value between the two ions' equilibrium potentials (Figure 4.10). In the process, however, the firing level of the postsynaptic membrane is reached (see Figure 4.6), the adjacent voltage-gated sodium channels open, and the action potential starts propagating

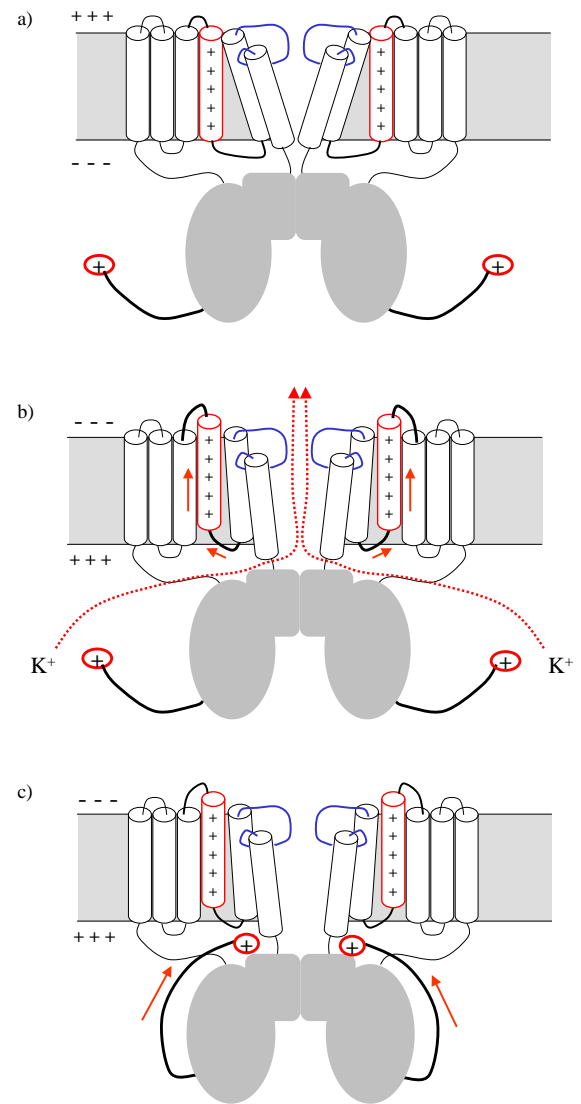


Figure 4.9. Structure and function of the voltage-gated potassium (K_v) channel. a: The resting state. The extracellular space (top) is electrically positive relative to the interior (bottom). The two gates - the positively charged transmembrane helix, and the flexibly attached N-terminal domain - are in their resting positions. b: Depolarization opens the first gate. The dotted arrows indicate the flow path of the K^+ ions. c: Inactivation occurs when the positively charged N-terminal inactivation peptide moves to block the entrance.

along the postsynaptic membrane in the usual way. We will see more about synapses in a later chapter.

Another principal means of action potential generation consists in spontaneous, rhythmic membrane depolarization. This occurs in specialized pacemaker cells in heart and smooth muscle. Therefore, while these tissues are modulated by neuronal and hormonal influences, they are capable of self-stimulation in the absence of any neuronal control⁴.

⁴Otherwise, heart transplants would not be possible (or at least would necessitate a simultaneous pacemaker implantation), since there is

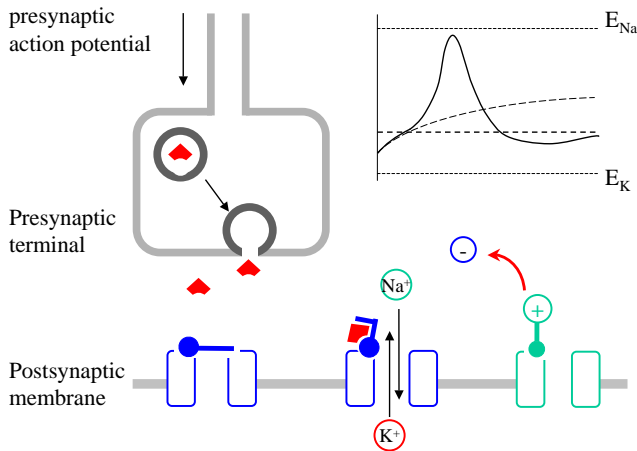


Figure 4.10. Overview of nerve impulse transmission in chemical synapses. The action potential in the presynaptic nerve cell induces release of the neurotransmitter (e.g., acetylcholine) into the synaptic cleft. The transmitter binds to its receptor, e.g. the nicotinic acetylcholine receptor (NAR). The NAR is a ligand-gated channel; it will open and become permeable to both K^+ and Na^+ . This will move the membrane potential toward the average of the two respective equilibrium potentials; however, in the process, the firing level of adjacent voltage-gated sodium channels will be exceeded, and a full action potential will be triggered (inset).

There are two major differences between action potentials that occur in nerve cells or skeletal muscle cells on the one hand, and in heart muscle cells on the other:

1. The duration of the action potential in the heart is much longer – several hundred milliseconds as opposed to several milliseconds in nerve and skeletal muscle. While each skeletal muscle contraction is triggered and sustained by a repetitive burst of many action potentials, in the heart there is only one action potential per heart beat.
2. While sodium is the major ion species responsible for excitation in nerve cells and skeletal muscle, in the heart pacemaker cells this role is taken by calcium. Calcium also has a prominent role in the excitation of smooth muscle cells.

Two types of calcium channels control the spontaneous formation of an action potential. These channels differ in their respective response to the prevailing membrane potential. One of them (the Ca_T channel) opens slowly but steadily at low potentials, thereby ramping up the membrane potential to the firing level. At this point, the Ca_L channel responds and induces rapid and complete membrane depolarization (Figure 4.11).

The heart also provides us with the ‘classical’ example of the third major way to trigger an action potential, which is

no way to immediately ‘wire’ the transplanted organ to the receiving patient’s nervous system.

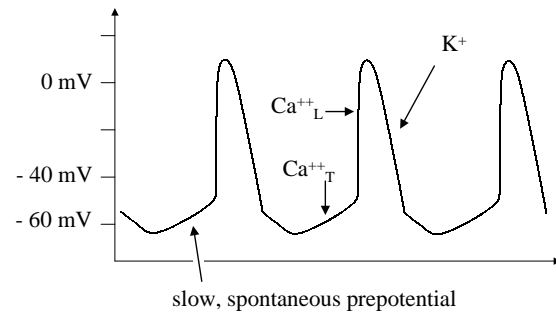


Figure 4.11. Generation of spontaneous action potentials in the cardiac conduction system. Depolarization starts as a slowly ascending prepotential that is due to the Ca_T channel. Once the corresponding threshold is reached, the Ca_L channel opens, and the action potential is triggered. It is terminated by inactivation of the Ca^{++} channels, and by the opening of K_V channels (which have the same role here as in the skeletal muscle and nerves).

by electrical coupling to a neighbouring cell via gap junctions (Figure 4.12). The excitation that is spontaneously generated in the small number of specialized pacemaker cells in the conduction system⁵ spreads in this way across the entire heart and ensures coordinated action. The speed of conduction varies in different parts of the heart, and the atria are excited and will contract before the ventricles. Groups of smooth muscle cells in many organs are likewise connected to each other and thus behave as functional units in a similar way.

4.5. Anion channels

All the ions we have covered so far are cations. What about anions? The major anion involved in electrical excitation is chloride. Figure 4.13 summarizes how it fits into the picture: Because of its opposite charge, chloride

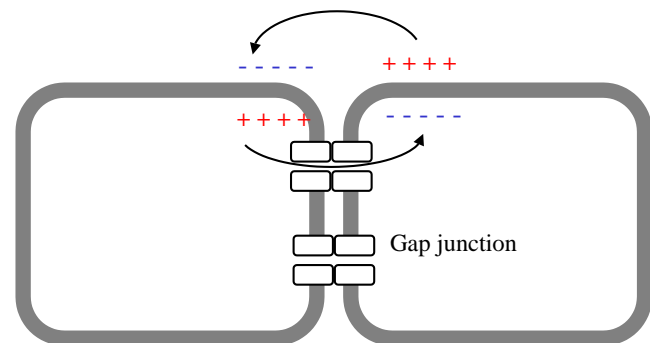


Figure 4.12. Gap junctions allow flow of ions between cells and therefore allow action potentials to spread.

⁵In a healthy heart, the primary rhythm is generated by the sinoatrial node. If the latter is damaged or disconnected from the subsequent parts of conducting system, lower centers such as the atrioventricular node or the bundle of His can take over and supply a somewhat slower rhythm.

actually opposes the excitatory action of Na^+ , although it closely resembles the latter in its distribution across the cell membrane (cf. table 4.1). The opening of chloride channels therefore will inhibit rather than promote membrane depolarization. Accordingly, chloride channels occur in *inhibitory* synapses within in the central nervous system. Inhibitory synapses do not trigger action potentials; they rather suppress their formation in nearby excitatory synapses that engage the same postsynaptic cell (see Figure 7.1). Such chloride channels are mostly actuated by the inhibitory transmitters glycine and GABA (γ -aminobutyric acid). GABA receptors are of central importance in the pharmacology of anaesthetic and sedating drugs.

(Notes)

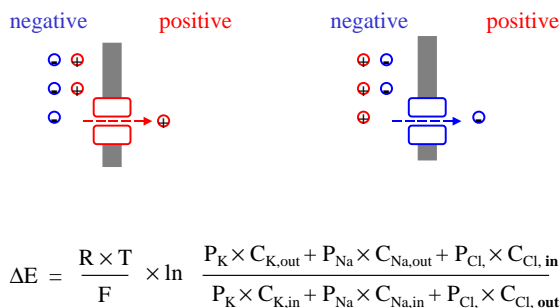


Figure 4.13. Top: Gradients of anions, because of their opposite charge, have effects on the membrane potential that are opposite to those of cations. Bottom: The Goldman equation for sodium, potassium, and chloride.

Chapter 5. Drugs that act on sodium and potassium channels

After discussing the basic principles of ion channel function in membrane excitation, it is important to note that for each major ion species there is a multitude of channels with specialized roles in different cell types and cell compartments. This is particularly striking in the case of K^+ and Ca^{++} channels. It was noted before that K^+ channels may be either constitutively open or controlled by electrical fields or ligand binding. These major functional classes are structurally different from each other; this is apparent already by comparing the number of transmembrane helices contained in each of the channel proteins (Figure 5.1). While K_V channels mediate the repolarization following an action potential, the basal K^+ permeability at the resting potential – the one that actually keeps the resting potential close to the K^+ equilibrium potential – is largely due to constitutively open ‘leak’ channels, which are part of a structurally distinct family (Figure 5.1). ‘Inward rectifiers’ are yet another family. We will see below that the sulfonylurea receptor, a membrane protein associated with the inward rectifier channel (K_{ir}), constitutes the main drug target related to the K^+ channels.

In addition, even within each individual family, there is a considerable number of variant genes. As an example, Figure 5.2 shows the homology tree for the K_V genes in the human genome. Additional variation arises from the fact that

the functional channel is a tetramer that can be composed of one or several types of subunits (Figure 5.2, right). In fact, several K_V genes have been shown not to yield functional channels when expressed alone, indicating that the proteins they encode may only function in heteromeric combinations with other K_V gene products. Most of the possible combinations are as yet uncharacterized with respect to occurrence and function.

Sodium channels occur in multiple subtypes, too, although they are not quite as varied as potassium channels are. The voltage-gated sodium channels do exhibit homology to K_V channels, but in contrast to the latter they are monomeric, i.e. the four functional subunits that are separate in the K^+ channels are merged into one polypeptide chain in the Na^+ channels. Calcium channels, in contrast, are a fairly varied group again, and include both voltage-gated and ligand-gated channels¹. The ligands are of very heterogeneous description and include nucleotides, proteins such as calmodulin and G proteins, and ions. With the ryanodine receptor channel, Ca^{++} occurs both as a conducted ion and as a channel regulator, causing a very peculiar behaviour of this channel.

Why harp on this multitude of channel subtypes? One important consequence is that it is very difficult at present to be sure about the spectrum of channels that may actually be targeted by a given drug *in vivo*. Observation of even strong interaction with one channel type in an *in vitro* mod-

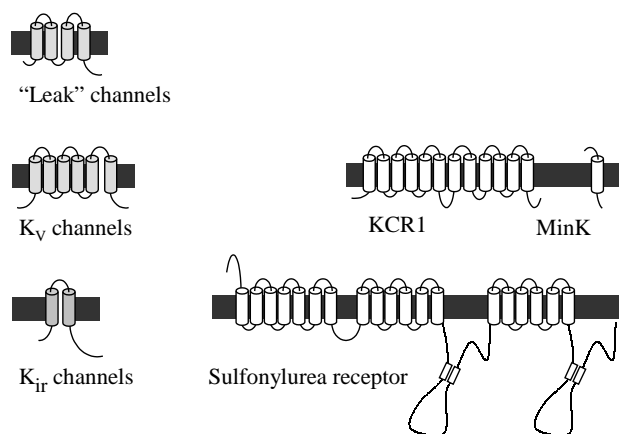


Figure 5.1. Types of potassium channels. Leak channels have four transmembrane helices per subunit, voltage-gated channels (K_V) have 6, and inward-rectifier channels (K_{ir}) have two. The activity of K_V channels is modulated by accessory proteins such as KCR-1 and MinK, whereas K_{ir} channels are controlled by the sulfonylurea receptor. Multiple subtypes exist for all types of channels.

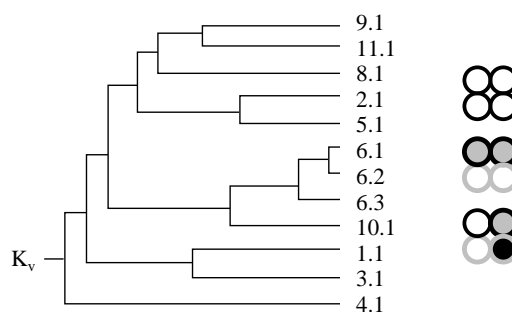


Figure 5.2. Variation of voltage-gated potassium channels. Left: Homology tree of voltage-gated potassium channels. Right: The variety of channels is considerably increased by mixing and matching of subunits within the tetramer.

¹And, to make matters a bit more complicated yet, many voltage-gated channels are modulated by ligands. Nature doesn't care too much about our neat abstractions.

el does not imply that this will be the only drug target in vivo, or even the most important one. Accordingly, with many drugs that act on ion channels, there is still a good deal of uncertainty with respect to their precise physiological mechanism of action.

Drugs that act on cation channels are used in various clinical applications. With sodium channels, these are:

- Cardiac excitation: Suppression of arrhythmia
- Neural conduction: Local anaesthesia
- Cerebral excitation and conduction: Suppression of epilepsy

Potassium channels are drug targets in the following contexts:

- Cardiac excitation: Suppression of arrhythmia (experimental)
- Vascular smooth muscle tone: Reduction of blood pressure
- Pancreatic β -cells: Enhancement of insulin secretion

Drugs acting on calcium channels have a similar range of applications:

- Cardiac excitation: Suppression of arrhythmia
- Vascular smooth muscle tone: Reduction of blood pressure

We will now look at drugs that manipulate Na^+ and K^+ channels. Drugs that target calcium channels will be covered after a bit more of theory in a later section.

5.1. Local anesthetics

Sodium channels are responsible for the propagation of action potentials in nerve fibers. Local anesthetics are blockers of sodium channels. They will thus intercept the propagation of action potentials along nerve fibers and in this way, among other things, prevent perception of pain.

We have seen before that drug receptors may be (in fact, typically are) allosteric molecules. This also applies to voltage-gated channels. With these, the force or energy required for transition from the resting to the active state

is normally provided not by ligand binding but by an electrical field. We have seen as well that drugs may interact differentially with the inactive state and the active state of a receptor. With voltage-gated channels, we actually have three different conformational states – they may be closed, open or inactivated. The functional effects of local anesthetics are related to their interactions with both the open and the inactivated states (Figure 5.3). Interestingly, with the local anesthetics of the lidocaine group, these two interactions can be assigned to two different moieties of the drug molecule. These two moieties are represented by aniline and by diethylamine, respectively (Figure 5.4).

Binding of a drug to a channel in its open state would be expected to obstruct the channel lumen to a certain extent, depending on the location of the drug binding site relative to the ion-conducting pathway (or channel lumen).

The effect of diethylamine (DEA) on the conductance of a single Na_v channel is depicted in Figure 5.5a. In the control trace, the channel can be seen to oscillate between two states of conductance, with currents of 0 and ~ 1 pA, respectively. A conductance of 0 would be expected for the closed and the inactivated states, respectively, whereas the conductance of 1 pA would represent the open state². This illustrates a very neat feature of the single channel recording techniques – they let us observe the discrete and stochastic nature of conformational changes of the proteins in a much more direct fashion than typically possible with other allosteric proteins (e.g., enzymes or hormone receptors). In the presence of DEA, open and closed state still alternate, but the conductance of the open state is reduced by about 40%, indicating a partial blockade of the channel³.

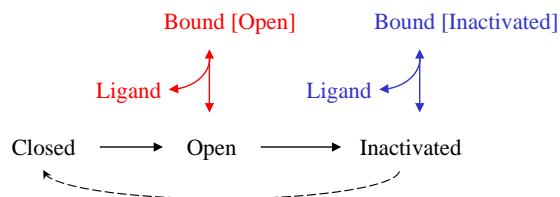


Figure 5.3. The three states of a voltage-gated Na^+ channel, and their inhibition by ligands binding to the open and inactive states, respectively.

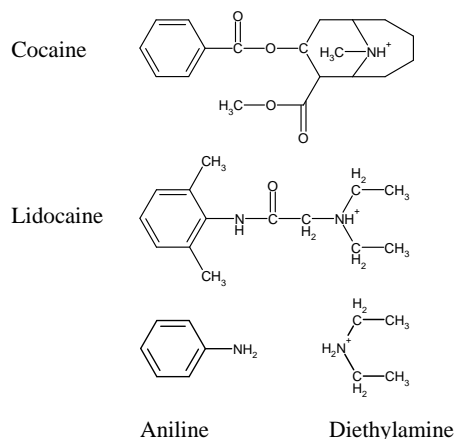


Figure 5.4. Structures of cocaine (the first local anaesthetic), the ‘standard’ local anaesthetic agent lidocaine, and the model compounds aniline and diethylamine.

²At constant voltage, the observed current will be a direct measure of the conductance. Of course, the conductance will be a characteristic of the particular channel in question, as will the rates of oscillation between the closed and open states.

Considering that DEA is a cation itself, it seems likely that it acts by binding within and direct obstruction of the channel lumen (although an indirect mode of inhibition – binding outside the lumen, causing obstruction in an allosteric fashion – cannot be ruled out).

A different pattern is observed with aniline (Figure 5.5b). Here, the conductance of the open state is unaltered; the effect of the ligand instead consists in the occurrence of extended time intervals with zero conductance (note that the time scale differs between Figure 5.5a and 5.5b), in line with the assumption that this ligand binds to and stabilizes the inactivated state of the channel. Interestingly, the channel also shows brief closing intervals that resemble those in the control trace. These might represent either direct reversions to the closed state, or inactivation events that revert to the closed (yet not inactivated) state before binding of aniline.

If the two moieties of lidocaine exert distinguishable effects on different states of the channel, what is the spatial relationship between the two binding sites? Consider the concentrations of aniline and diethylamine in the experiments above. They are very large – about 10^3 -fold higher than the binding constant for lidocaine ($12\ \mu\text{M}$). The higher binding affinity of lidocaine then suggests that the two lower affinities of its components may combine – which could only work if the two sites were adjacent to each other (Figure 5.6).

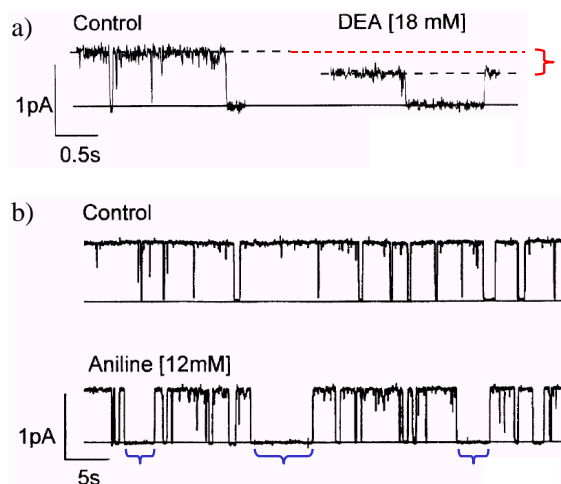


Figure 5.5. Single channel recordings of a Na_v channel in the presence of diethylamine (DEA) and aniline, respectively. See text for details.

³Binding of DEA would seem to be instantaneous, i.e. very fast with respect to the changes in channel conformation and the response time of the electrical detectors, so that the open state appears to be uniform; in reality, there should be an equilibrium and rapid alternation of ligand-bound and unbound states, as depicted in Figure 5.3



Figure 5.6. Spatial relationship of the binding sites for diethylamine and aniline, respectively, on the Na_v channel. Left: If the binding sites were distantly located, lidocaine should only be able to interact with one site at a time. Right: The substantially higher affinity observed with lidocaine as compared to its components suggests that the two sites are close to each other.

In Figure 5.3, we omitted the possibility of lidocaine interacting with the resting (closed) sodium channel. How do we know? Evidence of this is depicted in Figure 5.7c. It shows that the fraction of channels ready to open in response to a sudden depolarization depends on the level of the resting membrane potential before the depolarization. At levels below $-90\ \text{mV}$, there isn't much of a difference depending on whether or not lidocaine is present – we would get a maximum response regardless. At these very low membrane potentials (and sufficient time after the last opening inactivation cycle), all channels will be in the closed state and ready for opening (cf. Figure 5.3). If lidocaine were able to arrest channels in the closed state and prevent them from opening, we should see a reduced response from any starting level of the membrane potential⁴.

How can we explain the shift of the responsiveness of sodium channels to lower voltages? Figure 5.7a illustrates the kinetic law of reactivation; it is a simple first order process. If lidocaine is bound to the inactivated channel, the reactivation rate constant is decreased. In addition, the reactivation rate constant varies with the membrane potential. So does the rate constant of channel opening; however, the two vary in opposite ways, as illustrated in Figure 5.7b. At very low membrane potentials, reactivation will always be fast relative to opening, and the majority of channels will therefore be in the closed state, ready to open. As the potential increases, opening will get faster and reactivation slower, and somewhere above $-60\ \text{mV}$ the balance will tilt, to that the lifetime of the inactivated state now exceeds that of the closed state. This leads to a depletion of the closed state. Since lidocaine reduces the rate of reactivation at all membrane potentials (Figure 5.7b), the tipping of the balance will occur at lower values, so that at the physiological resting potential ($-70\ \text{mV}$) the closed state is already depleted, and no action potential will be triggered.

⁴This does not exclude the possibility that there may be some kind of silent, functionally inconsequential binding of lidocaine to the closed state. This could only be asserted by direct assays, e.g. using radiolabelled ligand.

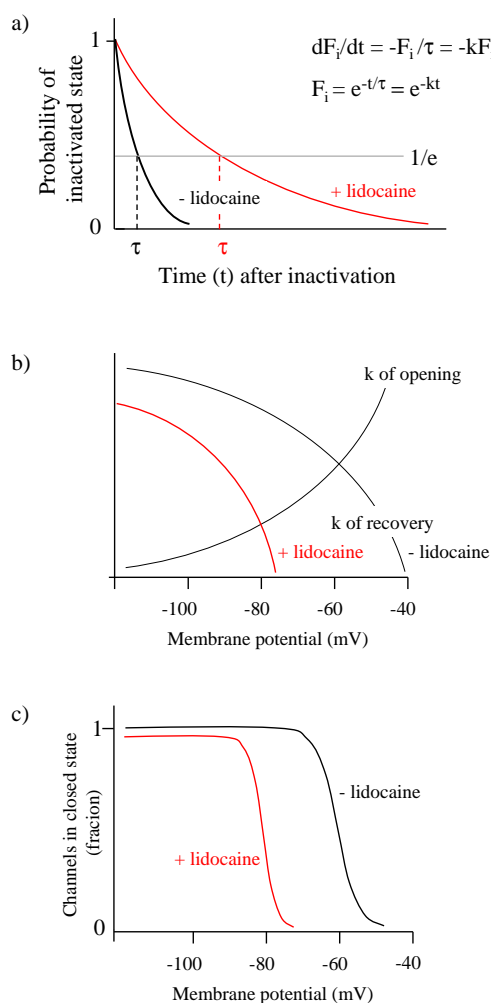


Figure 5.7. Inactivation of Na_v channels by lidocaine. a: First-order kinetics of reactivation. b: Variation of the rate constants for channel opening and channel reactivation with the membrane potential. c: Fraction of Na_v channels in the closed state (i.e., ready for opening in response to depolarization) as a function of the membrane potential. The diagrams are not drawn to scale.

It is interesting to note that, even in the absence of lidocaine, the number of responsive channels starts to drop between the resting potential (-70 mV) and the firing level (-55 mV). From this, we would expect a slow, partial depolarization to render the membrane refractory to excitation. This property of the Na_v channels may be important in a phenomenon called ‘depolarizing block’ in skeletal muscle cells. We will look into this in a later section.

Lidocaine is structurally similar to cocaine, which was the first clinically useful local anaesthetic (Figure 5.4). The stimulating effect of cocaine, however, is due to its effect on a second, different receptor in the brain that indirectly amplifies the effect of dopamine and norepinephrine (we will deal with this matter in a later lecture). This effect is actually observed at concentrations lower than those required for the blocking of sodium channels. Yet, local application of cocaine will result in very high concentrations that will

easily exceed the threshold for sodium channel blockade. Cocaine was first used in the eye, from where uptake into the systemic circulation is quite insignificant. This also seems to be its only remaining use in medicine. In other applications such as spinal and intravenous anaesthesia, which will lead to higher systemic drug concentrations and therefore potentially more side effects, lidocaine and similar drugs have replaced cocaine.

5.2. Sodium channel blockers as antiarrhythmic agents

A second major clinical application for lidocaine and related sodium channel blockers consists in the suppression of arrhythmias in the heart, which most commonly arise there as a consequence of some hypoxic tissue damage⁵. To understand this usage, we will briefly look at some details of heart physiology.

As noted before, the heart has its own conduction system for creating rhythmic excitations and propagating them in an orderly fashion to the muscle cells. The primary pacemaker is the sinoatrial node, which sits somewhere in the wall of the right atrium (Figure 5.8a). We have already seen before that it utilizes calcium and potassium but not sodium channels to create a spontaneous rhythm (Figure 5.8b, top). Specialized muscle fibers conduct each action potential first to the atrio-ventricular node and from there to the bundle of His, the Purkinje’s fibers and finally the muscle cells. The lower parts of the conduction system are capable of generating spontaneous rhythms, too, but slower ones; thus, in a healthy heart, their own rhythm gets overridden by the sinoatrial node. However, if the sinoatrial node fails, the rhythms of the downstream parts (typically the AV node; Figure 5.8b, center) will become manifest.

The ‘worker’ heart muscle cells (as opposed to the cells in the conduction system, which are also specialized muscle cells) are peculiar in using both Na^+ and Ca^{++} in the depolarization phase of the action potential (Figure 5.8b, bottom). While they do not normally create action potentials themselves, under pathological conditions some of them may show spontaneous discharge. This depolarization may then spread across the entire heart (or parts of it) and interfere with normal and regular activity. While both calcium and sodium channel blockers have their applications in treating heart arrhythmias, the beauty of the sodium channel blockers is that they will not interfere with the activity of the regular pacemakers (since those essentially don’t use sodium channels). Another beneficial feature was pointed out above: Lidocaine extends the duration of the inacti-

⁵Although it is interesting to note that there are hereditary arrhythmias due to mutations in ion channel genes.

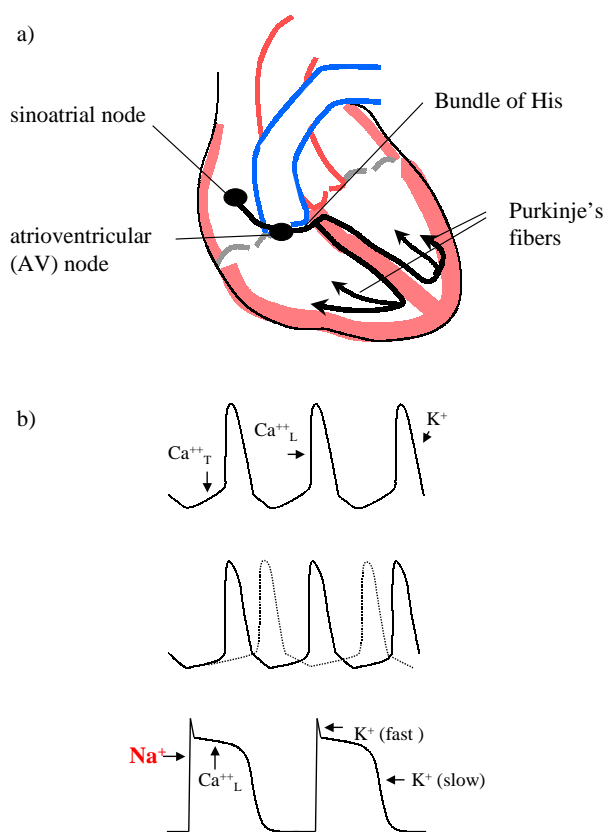


Figure 5.8. The conduction system of the heart. a: Anatomy. b: Electrical rhythm in the sinoatrial node (top), atrioventricular node (center), and the heart muscle (bottom). The dotted line in b (center) represents the own rhythm of the AV node that normally gets overridden by the faster sinoatrial rhythm (solid line).

vated state of the sodium channel more strongly at higher membrane potentials (Figure 5.7). Since the resting potential will typically be higher in diseased cells than in healthy ones⁶, this feature may confer some selectivity of the drug for the diseased, electrically unstable cells and help limit toxicity.

Since local anesthetics are applied locally and mostly needed for short times of action, their rate of systemic elimination doesn't matter too much. However, treatment of arrhythmias is usually prolonged and should be possible by oral therapy. Lidocaine gets metabolized very rapidly, and the bioavailability after oral ingestion is only ~3%. Metabolism consists in dealkylation of the tertiary amino group, and in cleavage of the amide bond (Figure 5.9); it can therefore be used only intravenously. Two derivatives that partially (tocainide) or entirely (mexiletine) avoid these metabolic modifications are shown as well. These drugs, expectedly, have higher bioavailability and can accordingly be used orally.

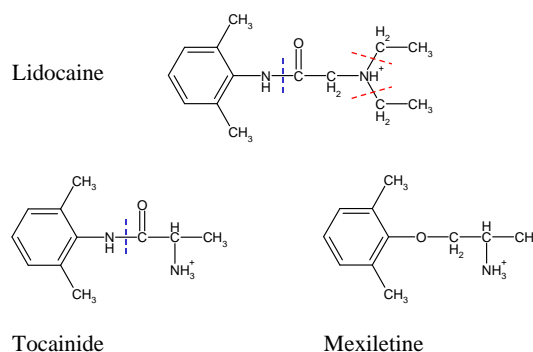


Figure 5.9. Structures and metabolically labile bonds of lidocaine, tocainide, and mexiletine.

Besides local anesthetics and their derivatives, there are other antiarrhythmic drugs that share their mechanism of action and block sodium channels. Quinidine is the enantiomer of quinine and, within a mixture with the latter, was initially used to treat malaria, before its anti-arrhythmic effects were noticed and utilized. Amiodarone is the most effective (and most toxic) anti-arrhythmic drug available. It seems to have a multitude of effects on a multitude of channels, and it is not known at present which single effect is the most significant one. Amiodarone is a good example of drug use on a largely empirical basis continuing in our era of purportedly entirely 'rational' medicine, without a clear understanding of the molecular mode of action.

5.3. Sodium channel blockers in epilepsy

Another field of application for sodium channel blockers is epilepsy. While epileptic seizures are a diverse and complex phenomenon, a key feature consists in bursts of excess excitatory activity of neurons in the brain. Sodium channel blockers will reduce nerve cell excitability, and they are thus one of the mainstays of anti-epileptic treatment. As but one example out of many⁷, Figure 5.10 shows phenytoin (or diphenylhydantoin).

Properties of phenytoin are

- good penetration of blood brain barrier;

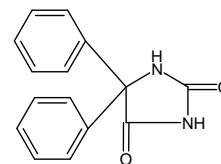


Figure 5.10. Structure of phenytoin

⁶ Consider that maintenance of the ion gradients costs ATP, and heart disease usually means disturbed perfusion; cells short of oxygen will have lower ATP levels.

⁷ Future medical students, look forward to delightful hours of memorization – which drug by what route in what type of seizure in patients of what age, gender, and zodiac.

- action on several cation channels besides Na_v . The contribution of these to the therapeutic effect is unsettled;
- strong enzyme induction (hepatic metabolism, CYP3A3). This gives rise to multiple drug interactions.

These characteristics are quite common among antiepileptic drugs.

5.4. Potassium channel blockers

Among the potassium channels, it is not the voltage-gated ones but a ligand-gated channel that constitutes the main drug target. This is the ATP-sensitive ‘inward rectifier’ (K_{ir}) channel⁸. The ATP sensitivity is conferred by a second membrane protein with which it is associated, the so-called sulfonylurea receptor (Figure 5.11a). The sulfonylurea receptor is homologous to the family of ‘ABC’ (ATP-binding cassette) transporters that occurs in both prokaryotes and eukaryotes. These proteins mediate the ATP-driven membrane transport of a wide variety of substances, including the extrusion of toxic compounds⁹. The sulfonylurea receptor, however, serves a different purpose: The conformational change induced by binding of ATP is relayed to the K^+ channel, which thus becomes responsive to ATP: High levels of ATP inhibit the channel, while lower levels cause it to open. This is schematically depicted in Figure 5.11b.

The sulfonylurea receptor does not only occur in nerve and muscle cells but also in pancreatic β -cells. These are located in the islets of Langerhans, and they function as both sensors of glucose and as purveyors of insulin. Insulin secretion is triggered by membrane depolarization, so that these cells are in fact excitable cells. The control of insulin secretion, and the role of the K_{ir} channel in it are depicted in Figure 5.12.

The ‘message’ conferred by insulin is that glucose is plentifully available, and that accordingly all cells should increase their uptake, glycogen stores should be replenished, and internal glucose production (gluconeogenesis) should shut down. Accordingly, a high level of blood glucose should stimulate insulin secretion. This is accomplished as follows: High blood glucose will raise the level of glucose in the β -cell. Glucose breakdown will raise the level of ATP, and the subsequent closure of the K_{ir} channel will induce membrane depolarization. This, in turn, will open voltage-gated Ca^{++} channels, and the increase in intracellular Ca^{++} will trigger exocytosis of insulin stored in secretory vacuoles. The same mechanism of coupling between mem-

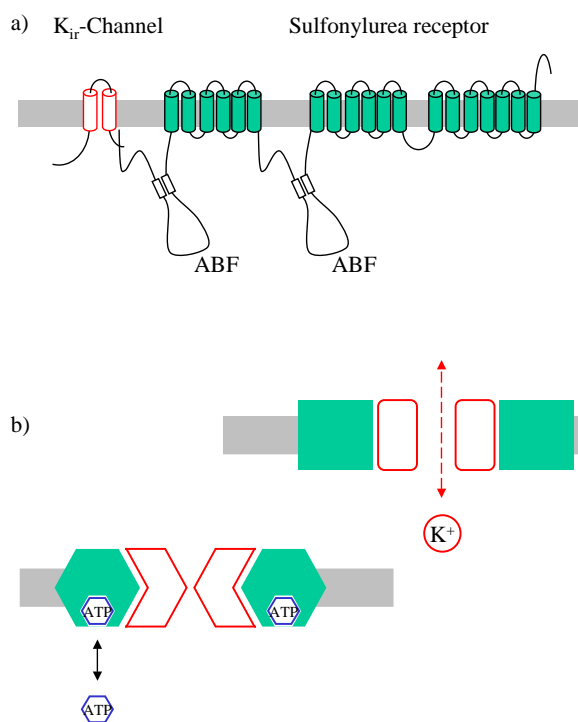


Figure 5.11. Structure and function of the K_{ir} /sulfonylurea receptor complex. Top: Both proteins have multiple membrane-spanning helices. The sulfonylurea receptor has two ATP binding folds (ABF). b: Binding of ATP to the sulfonylurea receptor closes the K_{ir} channel.

brane depolarization and exocytosis applies to the release of neurotransmitter in a synapse in response to a presynaptic action potential (see later).

Sulfonylurea derivatives such as tolbutamide (Figure 5.14) bind to the sulfonylurea receptor and thereby promote the closing of the K_{ir} channel. This amplifies the secretion of insulin – a desirable effect in type II diabetics, who suffer not from an absolute lack of insulin (as type I diabetics do) but from an inadequate response to it. Sulfonylurea drugs can be used orally, which is more convenient than insulin therapy. It is also less prone to result in excessive insulin action (hypoglycemia), since the physiological signal is amplified but not substituted. However, commonly the β -cells ‘wear out’ eventually, and insulin therapy has to be instituted anyway.

Another, as yet experimental use of sulfonylurea derivatives targets the K_{ir} channels in the heart in certain forms of arrhythmias. The sulfonylurea receptors in the heart are somewhat different from those in the β -cells, which makes selective drug action possible. Closing potassium channels should *increase* the excitability of heart muscle cells – in contrast to the effect of sodium (and calcium) channel blockers! Don’t worry, though – the cardiologists will find a way to show us why this is a good thing, like old farm-

⁸ ‘Rectifying’ here means that the conductivity varies somewhat with the orientation of the membrane potential. It is not fundamental to the function of this channel.

⁹ Some such proteins are up-regulated together with drug-metabolizing cytochrome P450 enzymes.

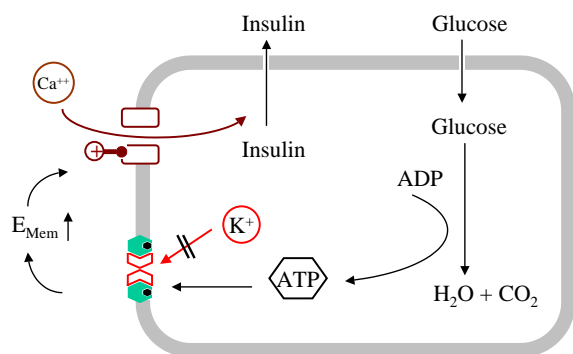


Figure 5.12. Regulation of insulin secretion by glucose in the pancreatic islet β -cell. An increase in glucose will yield increased ATP levels, which in turn will close the K_{ir} channels and partially depolarize the membrane. Opening of voltage-gated Ca^{++} channels will trigger exocytosis of insulin.

ers have a saying to predict each kind of weather in retrospect.

5.5. Potassium channel openers

Another practically important class of drugs that also interact with the sulfonylurea receptor are potassium channel openers. In keeping with our expectations, they will reduce membrane excitability. These are mostly targeted at the K_{ir} channels in the vascular smooth muscle cells (Figure 5.13).

A reduction of smooth muscle tension in the vessel walls will reduce the blood pressure, and high blood pressure accordingly is the usual rationale for their use. Their binding sites on the receptor molecule may be the same as those of the sulfonylurea derivatives, or not; correspondingly, they may or may not resemble the latter drugs in structure (Figure 5.14). The similarity is apparent with diazoxide. This drug actually also affects the pancreatic β -cells, and accordingly brings about reduced insulin secretion and elevated

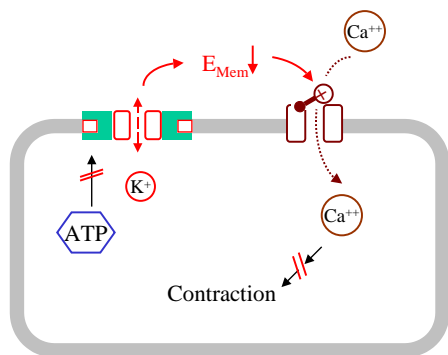


Figure 5.13. K_{ir} channel openers inhibit the excitation and contraction of smooth muscle cells. They act on the sulfonylurea receptor to open the channel, hyperpolarize the membrane, and reduce the uptake of Ca^{++} through Ca_v channels.

blood glucose, rendering it unsuitable for long-term use. The second drug shown (minoxidil) does not have this side effect. It is interesting for two reasons:

1. It is a 'pro-drug' – it is not minoxidil itself that mediates the pharmacological effect but minoxidil sulfate, which is formed in the liver by a sulfotransferase (the sulfate will replace the oxygen, Figure 5.14) – an example of an active drug metabolite. Relying on drug activation usually adds some variability to the clinical effect of a drug (there may be competing metabolic pathways ...), so one would normally prefer a drug that is immediately active. Can you see a reason why one would still prefer minoxidil over the sulfate?
2. One of the side effects of minoxidil is 'hypertrichosis', meaning increased hair growth, and consequently many patients refuse it. Strangely, however, the very same side effect has proven highly popular with some patients. Local application of the drug to balding areas is reportedly effective, too, although the beneficial effect ceases with discontinuation.

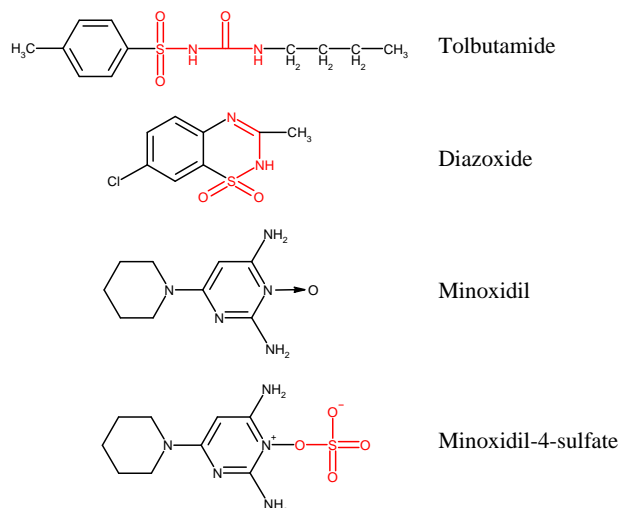


Figure 5.14. Structures of the K_{ir} channel openers diazoxide and minoxidil, and the active metabolite minoxidil sulfate. Tolbutamide (which blocks the channel) is shown for comparison.

(Notes)

Chapter 6. Some aspects of calcium pharmacology

Calcium has a dual role in the regulation of cell function: It carries charge and thus contributes to the changes of membrane potential in excitable cells, and it acts as a biochemical messenger by directly binding to proteins and modifying their functional state. Proteins directly or indirectly affected by calcium are very diverse and include enzymes, cytoskeletal proteins, ion channels, and transcription factors.

The level of free calcium in the cytosol is affected by multiple mechanisms of active and passive transport. These are summarized in Figure 6.1. While the level of calcium in the cytosol varies in time, it is always much lower than in the extracellular space, or than in the mitochondria and ER. These two organelles function as intracellular buffers or reservoirs of calcium. Accordingly, calcium transport systems operating in both directions exist not only at the cytoplasmic membrane but also at the mitochondrial (inner) membrane and the ER.

Many of the physiological effects of Ca^{++} are mediated by the small protein calmodulin (CaM), which upon binding of Ca^{++} changes conformation and binds to multiple effector proteins, including a variety of protein kinases; protein phosphorylation is a widely used means of regulation in signal transduction. Another protein directly activated by Ca^{++} is the protein phosphatase calcineurin, which will participate in the reversal of protein phosphorylation. The spectrum of target proteins for calcium-dependent protein kinases and phosphatases expressed in a given cell will vary and decide on the ultimate effects of calcium regulation in the cell¹.

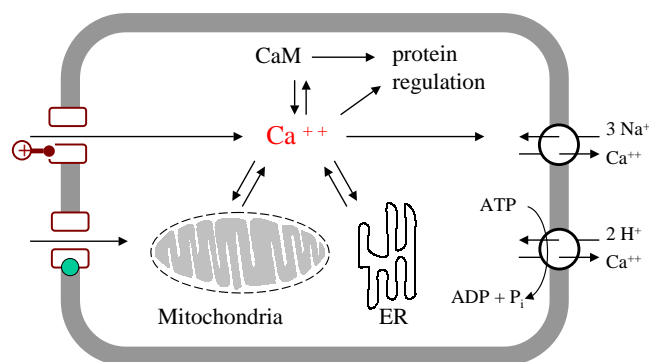


Figure 6.1. Overview of cellular calcium transport. Calcium enters the cell through voltage- or ligand-gated channels (left). It is extruded by ATP-driven pumps or by sodium antiport (right). Both the mitochondria and the endoplasmic reticulum serve as intracellular calcium stores. The cytosolic concentration is kept at ~100 nM under resting conditions. CaM: Calmodulin.

Calcium-dependent processes relevant to pharmacological intervention mainly include pace-making in the heart, and contraction in heart and smooth muscle. These are affected by drugs that either act on calcium channels directly, or on other receptors that will have some downstream effect on the cytosolic availability of calcium. Examples are the β - and α_1 -adrenergic receptors (see below).

6.1. Calcium in muscle cell function

Calcium has a pivotal role in the control of muscle cell action. Muscle cells occur in different types:

- Smooth muscle cells. They occur in hollow organs, including blood vessels,
- Heart muscle cells,
- Skeletal muscle cells.

Heart and skeletal muscle together are classified as striated muscle yet do have some important functional differences (see below). The muscle cells in vessel walls control the blood pressure, which makes them important drug targets; that heart muscle cells are important targets, too should go without saying.

We will first look at the role of calcium in the contraction of striated muscle. Figure 6.2a shows a light-microscopic picture of heart muscle. The striations are oriented perpendicularly to the longitudinal axis of the cells. The borders between the individual heart muscle cells are bridged by gap junctions, which will ensure swift spread of excitation from one cell to the next. Skeletal muscle cells form long syncytia in which the excitation spreads even faster.

At electron microscopic resolution, the striations appear more complex (Figure 6.2b). They correspond to densely and regularly packed filaments of actin and myosin, each composed of numerous, linearly polymerized subunits². The finer striations visible in EM are due in part to additional structural proteins, and in part to zones of overlap between actin and myosin.

While actin and myosin are present and responsible for motility in essentially all cells, a peculiarity of the striated

¹At very high concentration, Ca^{++} will trigger apoptosis (programmed cell death) in many cells. Thus, if a cell is rendered permeable to calcium by some pore-forming protein, it will commit suicide – a principle widely used by the immune system (complement, perforin), as well as microbes, which frequently secrete pore-forming protein toxins.

²The bonds between the ‘polymer’ subunits are non-covalent, however.

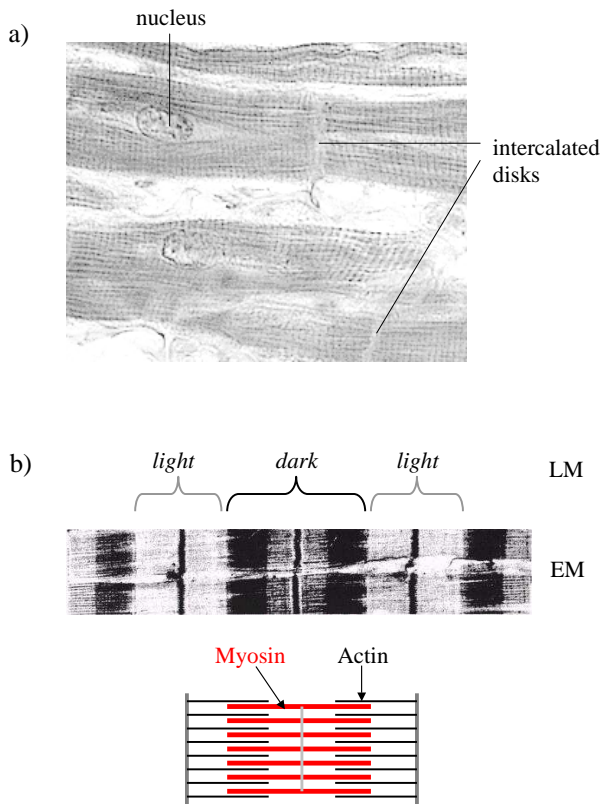


Figure 6.2. Ultrastructure of heart muscle. a: Light microscopy. Striations run perpendicularly to the cell longitudinal axis. Junctions between individual cells ('intercalated disks') are visible as slightly lighter bands; they contain numerous gap junctions. b: Electron microscopy (EM), and correlation to light microscopy (LM) and spatial arrangement of actin and myosin.

muscle (apart from the sheer amount and regular, parallel packing) is the presence of two additional proteins associated with the actin filaments. These proteins are troponin and tropomyosin, and they are crucial in the control of contraction.

The arrangement and the workings of actin, myosin, troponin and tropomyosin in striated muscle are summarized in Figure 6.3. The myosin heads are in close proximity to the actin filaments, but in the resting state direct contact between actin and myosin is blocked by the tropomyosin filaments. Upon cell excitation, calcium becomes available and binds to troponin, which in turn moves the tropomyosin out of the way. The heads of myosin are allowed to access their binding sites on actin. Binding causes the myosin head to bend. This will both propel the myosin filament along the actin 'track' and trigger the intrinsic ATPase activity of myosin. The energy of ATP cleavage is used to power cycles of binding, bending and release. This activity is commonly likened to rowing; the myosin heads, then, comprise both the oar and the biceps, whereas the actin filament is merely the water. Interestingly, the ATP is actually ex-

pended at the stage of the 'push' rather than 'pull' – which is where the analogy ends.

In striated muscle, the sheer amount of filaments is such that we actually need quite a bit of calcium to swiftly saturate the troponin molecules and trigger contraction. The lion's share of this calcium is not obtained from the extracellular space (via the voltage-gated Ca^{++} channel, the dihydropyridine receptor – see later) but from the intracellular storage, more specifically from the endoplasmic reticulum, which somebody found necessary to christen 'sarcoplasmic' reticulum in the muscle cell (gr. sarx, sarkos = flesh). It is released from there by a specialized Ca^{++} channel, the ryanodine receptor (RyR)³. This channel is activated by cytosolic calcium, which of course creates a fast and powerful

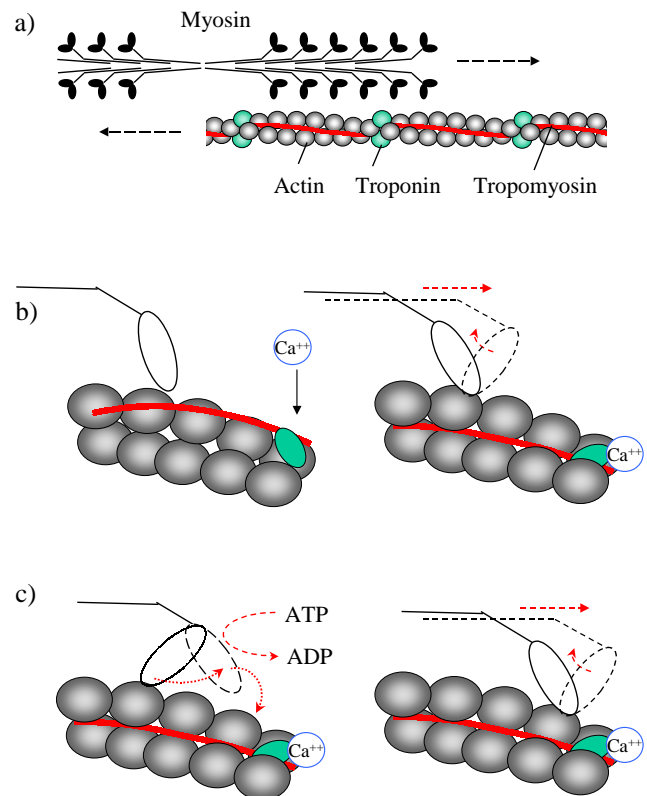


Figure 6.3. Structure and function of myofilaments. a: Arrangement of proteins within the filaments. b, c: Mechanism of myofilament motion. Calcium binds to troponin, which in turn causes tropomyosin to move and expose the myosin binding site on actin. Binding to actin causes the myosin heads to kink, which translates into a sliding motion. c: The kinked conformation of myosin cleaves ATP. In the process, myosin releases itself from actin and returns to the extended conformation; it then binds to another actin monomer, and the cycle is repeated.

³Ryanodine is a plant alkaloid that reduces the activity of the RyR and, accordingly, reduces the contractility of muscle. No medical use exists for this molecule, but it has been used for the affinity purification of the receptor.

amplification mechanism for the release of calcium from the ER and, thus, for contraction (Figure 6.4).

The anatomical arrangement of the cell membrane, the SR, and the myofilaments in the striated muscle is further optimized for rapid action. Figure 6.5 illustrates this for a skeletal muscle cell. The cytoplasmic membrane there forms invaginations called T (transversal) tubules, which protrude into the cell interior and enter into immediate contact with the SR cisterns, which in turn are aligned to the contractile filaments.

The close alignment of cytoplasmic and ER membranes is, in fact, crucially important for the workings of excitation-contraction coupling in the skeletal muscle. In these cells, we have a unique mechanism of activation of one channel by another: The RyR is directly hooked up to a cytosolic loop of the dihydropyridine receptor (DHPR; Figure 6.6a, b). Membrane depolarization will cause a conformational change to the DHPR, which in turn is directly and mechanically transmitted to the RyR, so that both channels open synchronously. This even works in the absence of any calcium flux across the cytoplasmic membrane – experimentally, skeletal muscle cells can be induced to contract in calcium-free buffers.

Although the anatomical relationships are closely similar in the heart muscle, there is no direct coupling between the DHPR and the RyR in this case (Figure 6.6c). Therefore, excitation-contraction coupling in the heart muscle cells *does* require flux of calcium across the DHPR channel, even if only as a trigger, which will then activate the RyR and release the lion's share of the calcium needed for activating troponin from the SR. Thus, drugs that reduce flow of Ca^{++} through the DHPR channel will reduce the force

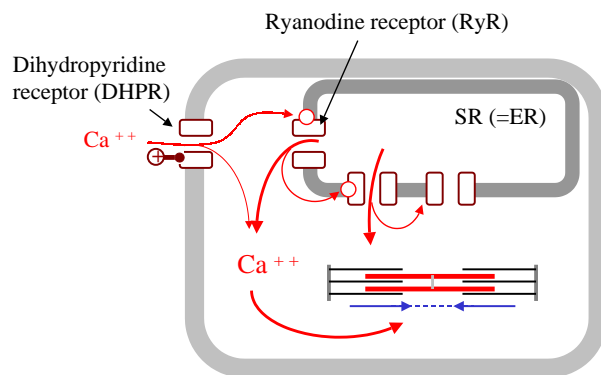


Figure 6.4. Excitation contraction coupling in heart muscle. Influx of extracellular Ca^{++} through the voltage-gated anesthetic receptor opens the ryanodine receptor in the sarcoplasmic reticulum (SR), which releases the bulk of Ca^{++} required for myofilament activation. Opening of ryanodine receptors by Ca^{++} will spread like a wave along the SR membrane – a kind of chemical membrane excitation.

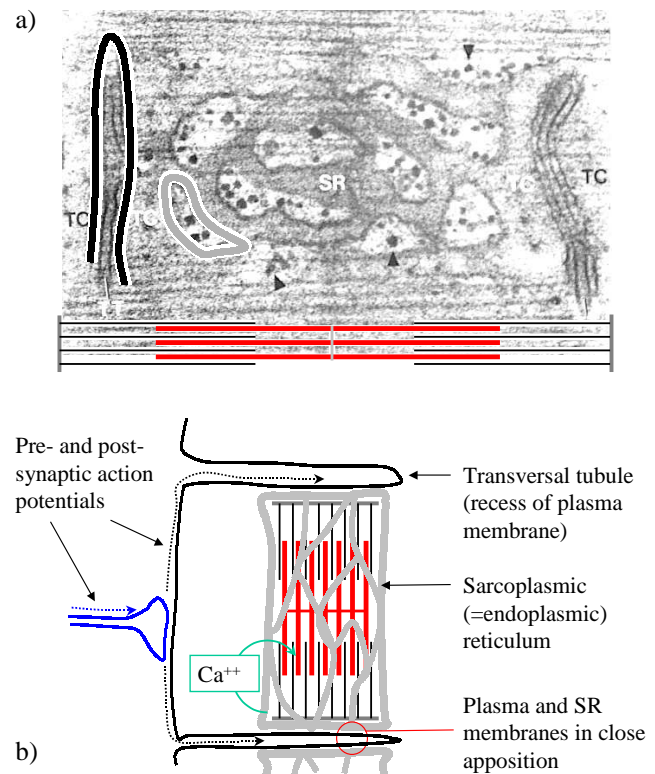


Figure 6.5. Anatomical relationship of transversal tubules, sarcoplasmic reticulum, and myofilaments. a: Electron microscopy. Colours of highlighted membrane structures as in b. b: Schematic. The transversal tubules are contiguous with the cytoplasmic membrane; they conduct the postsynaptic action potential to the cell interior, where it triggers the release of Ca^{++} from the sarcoplasmic reticulum. Circle: Zone of immediate apposition of the plasma and SR membranes. The dihydropyridine receptor channels are located here.

of contraction in the heart (and in smooth muscle, see later) but not the skeletal muscle.

6.2. Calcium channel blockers

Examples of such calcium channel blockers are shown in Figure 6.7. While nifedipine contains the dihydropyridine moiety (in red) after which the receptor has been named, verapamil does not, indicating that the receptor's name does not reflect any fundamental requirement of drug molecular structure.

We have seen before that calcium channels are not only important in the contraction of the heart muscle but also in the generation of cardiac rhythm. The DHPR is present in both the pacemaker and the muscle cells of the heart muscle. This means that calcium channel blockers will not only reduce the contractility of the heart muscle cells but also slow down the rhythm⁴. Slowing down the rhythm means

⁴ Verapamil is indeed commonly used to suppress certain kinds of cardiac arrhythmias.

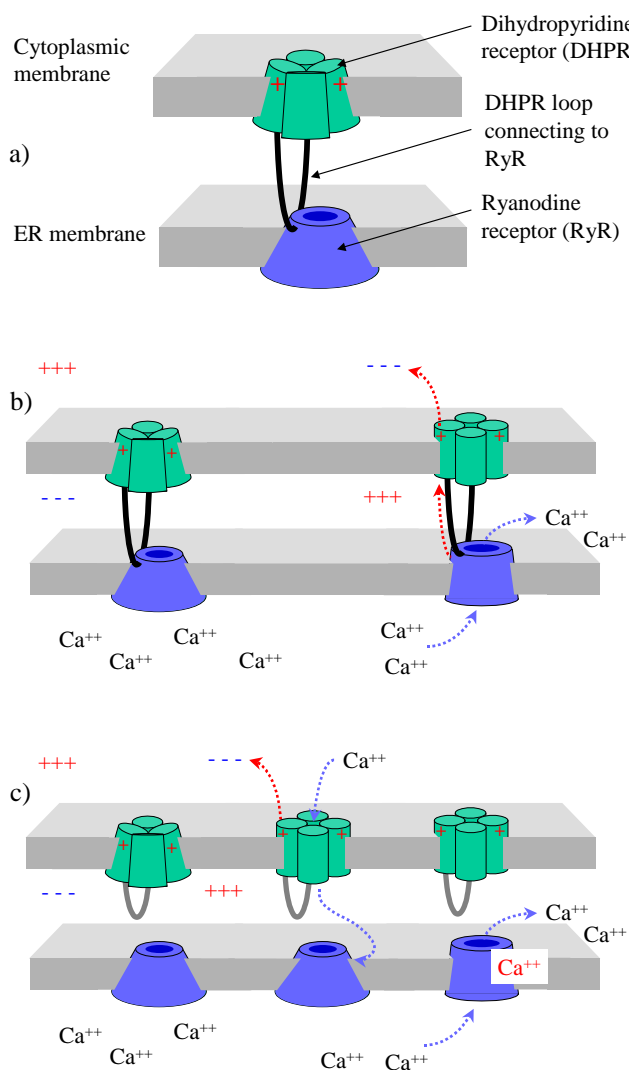


Figure 6.6. Excitation contraction coupling in skeletal muscle (a, b) and heart muscle (c). In skeletal muscle, the anesthetic receptor (DHPR) and the ryanodine receptor (RyR) are in direct contact (a). The conformational change to the former that occurs in response to membrane depolarization is sufficient to induce Ca^{++} release from the SR; a flow of Ca^{++} across the plasma membrane is not necessary (b). In heart muscle, however, this direct link does not exist, and Ca^{++} must therefore enter through the DHPR first (c).

increased duration of the interval between two contractions (the diastole). The diastole is the only period during which the pressure within the heart tissue does not exceed the arterial blood pressure; therefore, only during the diastole the heart tissue itself is effectively perfused. Reduced contractility and prolonged pauses combine to improve the metabolic situation of heart muscle cells suffering from reduced perfusion in occlusive disease ???

The DHPR is also found in vascular smooth muscle. While the mode of action of Ca^{++} is somewhat different in smooth muscle than in striated muscle (cf. Figure 6.10b, below), it is still crucial for contraction, and as in heart muscle,

flow of Ca^{++} across the plasma membrane is required. The DHPR in smooth muscle therefore constitutes an important drug target for calcium channel blockers as well, as they will decrease the vascular wall tension and, hence, the blood pressure. The DHPR subtypes in heart and smooth muscle differ, and some blockers act more strongly on either the smooth muscle (nifedipine) or the heart (verapamil).

6.3. Digitalis (foxglove) glycosides

In other (mainly elderly) patients, the main problem may consist not so much in under-perfusion of the heart muscle but in a weak contractility. The heart becomes distended, further reducing the effectiveness of contraction⁵. Here, we clearly want to increase the contractility of the heart muscle. The most effective way to do this is to raise the availability of calcium in the cytosol. This is done with digitalis (foxglove) glycosides. The mechanism of action of these is outlined in Figure 6.8a. Digitalis glycosides bind to and block the Na^+/K^+ -ATPase in the cytoplasmic membrane. The backup of sodium in the cell reduces the active export of Ca^{++} from the cytoplasm. Although the transfer of calcium across the cytoplasmic membrane is small relative to the fluxes occurring across the ER membrane, the ER stores will eventually fill up, and the cytosolic Ca^{++} level will be raised. This treatment is quite effective and was readily recognized as such in an earlier era that did not make use of advanced statistical methods to measure therapeutic responses. Nevertheless, the usage of digitalis is not undisputed, particularly in North America. Why? It is a matter of perspective. Patients usually benefit as long as they live – but they do not live longer. Digitalis glycosides have, as you will now have come to expect, effects on the pacemak-

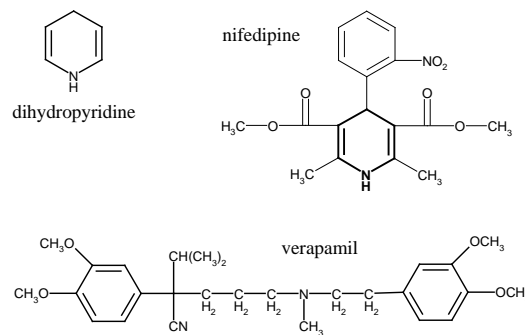


Figure 6.7. Structures of the dihydropyridine receptor Ca^{++} channel blockers nifedipine and verapamil. Only nifedipine actually is a dihydropyridine derivative.

⁵Recall that, in a closed container, the wall tension required to achieve a certain internal pressure grows with the diameter. Car tires have thicker walls yet hold lower pressure than bicycle tires.

er cells as well, and so they do promote certain types of arrhythmias themselves⁶.

If we consider the mode of action of digitalis, what would we expect the therapeutic index of the drug to be: Large or rather smaller? Complete receptor saturation will completely knock out the Na^+/K^+ -ATPase and thereby terminate the life of the target cell. Thus, it is quite obvious that we will have to walk a fine line in determining the right dosage. Therefore, we will have to consider very carefully the pharmacokinetic properties of the drugs, and the kidney and/or liver functions of the patient.

Figure 6.8b compares the three major digitalis glycosides that are (or have been) used clinically. The structure of digitoxin is depicted completely, with both the steroid-like ‘aglycone’ moiety and the three residues of digitose, which is a hexose that lacks two hydroxyl groups. These are represented by ‘R’ in the two other structures and actually not

required for activity – they can be replaced by, e.g., a single acetyl residue. However, the lactone group – the five-membered ring at the top – is essential. Its hydrolysis (cleavage of the bold single bond) or its reduction (of the bold double bond to a single bond) will abolish activity. And here is a snag: Digitoxin, being highly protein-bound, is not efficiently eliminated in the kidneys but instead conjugated in the liver and largely secreted into the bile and intestine. There, a large fraction undergoes cleavage of the (glucuronide) conjugate and then reuptake, i.e. enterohepatic cycling, so that this drug has a very long overall half-life. However, during the intestinal passage, the lactone ring may be reduced by bacterial enzymes, and the drug molecule thus be inactivated. Due to the individual variations in the intestinal flora, this reduction may occur to varying extents, which introduces an element of variability into the effectiveness of this drug. Worse still, the analytical separation of the reduced and the unreduced drug is not trivial and is not achieved by the routine drug monitoring assays presently available.

Digoxin has one additional hydroxyl group, which (some-what miraculously, it would seem) changes its pharmacokinetic parameters such that it is largely eliminated in the kidneys. This avoids the intricacies of entero-hepatic cycling; however, it renders the rate of elimination dependent on kidney function, which tends to be more variable than liver function. Also, intestinal uptake directly after oral ingestion tends to be lower and more variable. An even more polar derivative is ouabain. This drug is rapidly eliminated in the kidneys. However, it is not efficiently taken up after oral application and so can only be used intravenously.

Recently, it has been found that ouabain (once thought to occur in plants only) is, in fact, secreted by the adrenal glands – at concentrations far lower than those necessary for inhibition of the Na^+/K^+ -ATPase. Nevertheless, there is experimental evidence that even at the low physiological concentrations, ouabain may modulate cellular function. This is illustrated in Figure 6.9. While half-maximal inhibition of Na^+/K^+ -ATPase occurs at about 100-200 μM (Figure 6.9a), elevations in the cellular calcium level (measured using a cell-permeant, calcium-binding fluorescent dye) are detected with nanomolar range concentrations of ouabain. Remarkably, these occur as slow waves rather than continuously. In the study cited, a secondary effect on a certain transcription factor was noted as well. While the physiological role of endogenous ouabain remains unsettled, the occurrence of both temporal and spatial waves and spikes of calcium signals is increasingly recognized, and adds yet another layer of complexity to its role in cellular signalling.

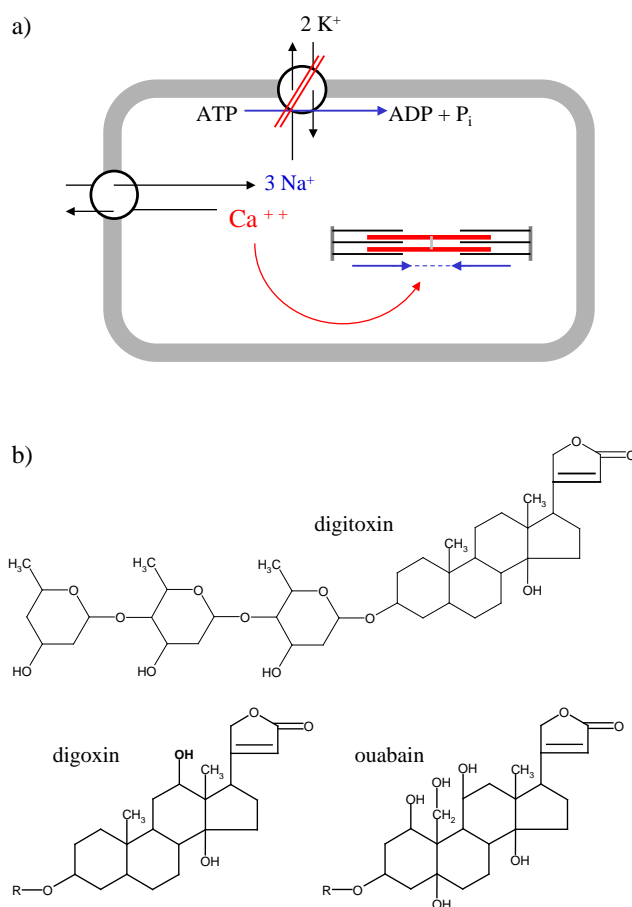


Figure 6.8. Digitalis (foxglove) glycosides. a: Mode of action. The drugs inhibit the Na^+/K^+ -ATPase. The increased intracellular sodium concentration will reduce the activity of the $\text{Na}^+/\text{Ca}^{++}$ antiporter system and therefore lead to an increase of intracellular Ca^{++} and augment myofilament contraction. b: Structures.

⁶On the other hand, with other types of arrhythmias, digitalis may be used primarily for the sake of its action on pacemaker cells.

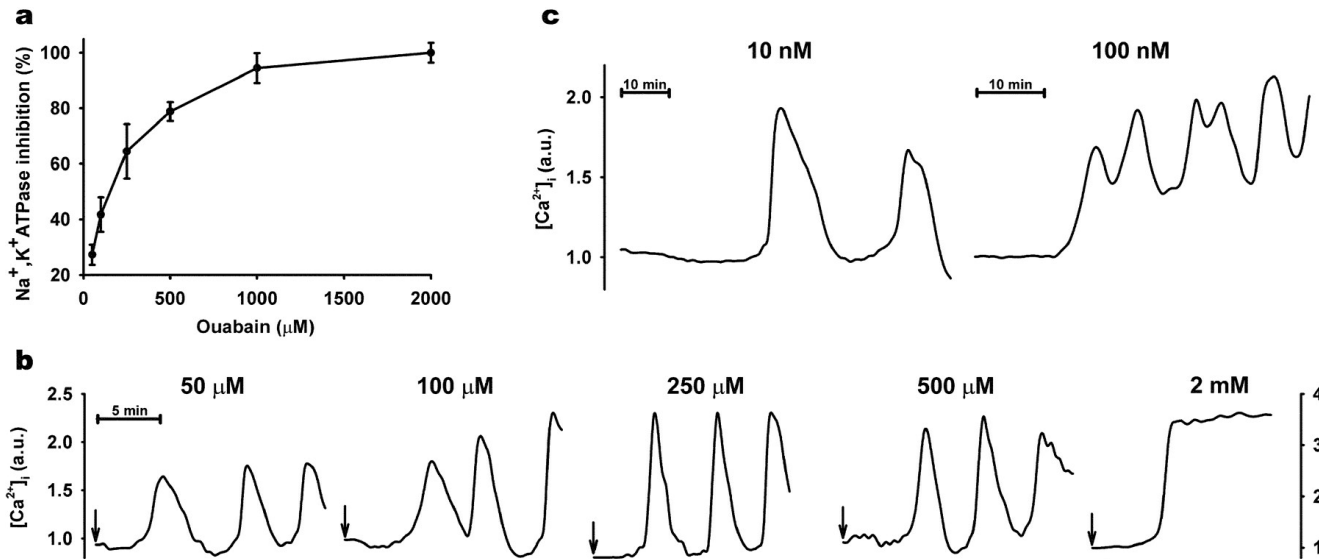


Figure 6.9. Oscillatory changes of intracellular Ca^{++} induced by low concentrations of ouabaine. **a:** Inhibition of Na^+/K^+ -ATPase, which is considered to be responsible for the therapeutic effect, requires micromolar concentrations. **b, c:** Slow, oscillatory changes occur at considerably lower concentrations, and the oscillatory pattern is sustained up to surprisingly high concentrations. From PNAS (2001), vol. 98: 13420-13424.

6.4. Calcium-dependent signaling by adrenergic receptors

Calcium is also involved in the cellular effects of mediators such as epinephrine and norepinephrine. In the heart, the predominant adrenergic receptors are of the type β_1 , and both agonists and antagonists of β receptors are being used in cardiac therapy. β -Adrenoceptors always activate adenylate cyclase and, through cAMP, protein kinase A (PKA). However, the downstream effectors may differ depending on the cell type. In the heart, PKA changes the activity of several target proteins including RyR, DHPR, and the regulatory ER membrane protein phospholamban (Figure 6.10a). While the effect of phospholamban phosphorylation is a disinhibition of SERCA (SR/ER calcium transporter, an ATP-dependent uniporter) that will tend to reduce cytosolic Ca^{++} , the first two will increase the availability of Ca^{++} , which seems to be the net effect⁷.

In smooth muscle, contraction is slower and longer lasting than in striated muscle. Regulation of actin and myosin does not work by way of troponin / tropomyosin but by phosphorylation of the regulatory myosin light chain (Figure 6.10b). This is catalyzed by myosin light chain kinase (MLCK), which is calmodulin-dependent and, hence, again under the control of calcium. However, less calcium is nec-

essary in this regulatory mechanism, because MLCK provides an extra amplification stage not present in the direct binding of calcium to troponin⁸.

In smooth muscle, β -adrenoceptors *decrease* contractility: PKA phosphorylates MLCK, which thereby becomes inactivated. In contrast, α_1 -adrenoceptors *increase* smooth muscle contractility. They activate phospholipase C, which in turn releases inositoltriphosphate (IP_3) from the endoplasmic reticulum by binding to a cognate receptor channel (Figure 6.10c). Ca^{++} then binds to calmodulin, which in turn activates myosin light chain kinase.

⁷Maximum efficiency of heart muscle action will require full relaxation alternating with full contraction. To this end, cytosolic Ca^{++} has to undergo cyclical changes, so that the increase of transport capacity in both directions is less strange than it might appear at first sight.

⁸Also, smooth muscle has less actin and myosin than striated muscle has (about 5 times). Nevertheless, the maximum force developed by smooth muscle per cross-sectional area is larger than that of striated muscle! Clearly, striated muscle is not optimized for force but rather for speed.

(Notes)

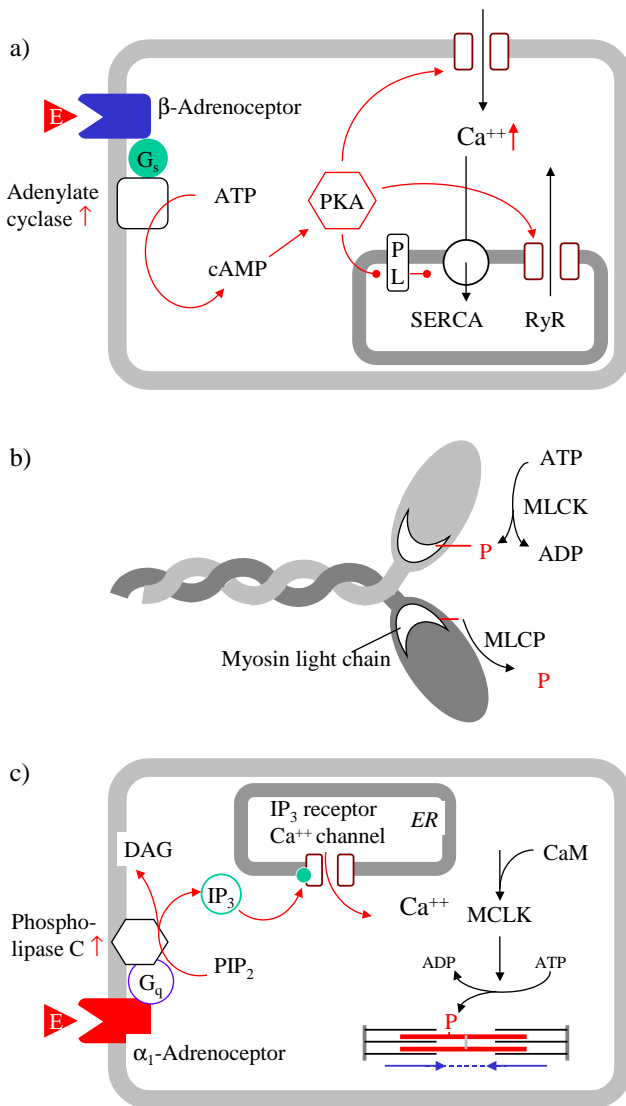


Figure 6.10. Calcium-dependent signalling by adrenergic receptors. a: β -Adrenergic receptors activate adenylyl cyclase. cAMP activates protein kinase A (PKA). In heart muscle, PKA phosphorylates several Ca²⁺ transporters and channels, so that the amount of Ca²⁺ available for contraction is increased. PL: Phospholamban; SERCA: SR/ER Ca²⁺ transporter. b: In smooth muscle, myosin activation in works by way of phosphorylation, which is performed by myosin light chain kinase (MLCK). Inactivation is accomplished by myosin light chain phosphatase (MLCP). c: α_1 -Adrenergic receptors stimulate phospholipase C, which releases inositoltriphosphate (IP₃). IP₃ binds to a cognate ligand-gated Ca²⁺ channel in the ER and releases Ca²⁺, which with calmodulin activates MLCK.

Chapter 7. Some aspects of neurophysiology relevant to pharmacology

This chapter presents some basic facts from neurophysiology that will be needed in subsequent chapters on the pharmacology of the nervous system.

The nervous system can be divided according to different categories:

1. Central versus peripheral. The central nervous system comprises the brain and the spinal cord, which together are protected from the periphery by the blood brain barrier.
2. Somatic versus autonomic. The somatic nervous system comprises functions that are conscious – conscious sensations such as touch, temperature, pain etc., and voluntary movements. Conversely, the autonomic nervous system deals with unconscious sensory input such as blood pressure, blood oxygen and carbon dioxide levels¹, and the likewise unconscious regulatory responses to it.

The two above distinctions are ‘orthogonal’, which means that we find autonomic and somatic parts in both the central and the peripheral nervous system. Among the four resulting categories, the peripheral autonomic system has a prominent role as a drug target, for the following reasons:

- It is responsible for the functional regulation of interior organs and physiological parameters (such as heart rate and blood pressure) that are relevant to disease.
- It is readily accessible, i.e. not protected by the blood brain barrier.
- As with all of the peripheral nervous system, its organization is comparatively simple, at least more so than that of the brain is, and selective drug action is more feasible.

The relative simplicity of the peripheral nervous system also explains why drug actions occurring there usually are understood in a more definite way. With centrally acting drugs, there often is a vagueness of understanding that makes them less compelling as examples for an introductory class – although in clinical practice they are very important, of course.

Selective drug action on the nervous system is feasible to the extent that different subsystems use specific transmit-

ters and receptors. Before we take a look at these, we will first review some general aspects of nerve cell structure and function.

Figure 7.1a shows the schematic of a nerve cell (neuron). The soma (greek: body) contains the nucleus and the bulk of the biosynthetic apparatus (ER etc.) It branches out into multiple dendrites (greek dendron = tree) and a single, usually longer axon that may branch in turn to reach multiple target cells. *Afferent* synapses are mostly located at the dendrites and the soma; they receive signals from other neurons. The input from all dendritic synapses is summed up and controls the *efferent* activity of the neuron. The latter consists in the generation of action potentials. The efferent action potentials travel swiftly down the axon to the ef-

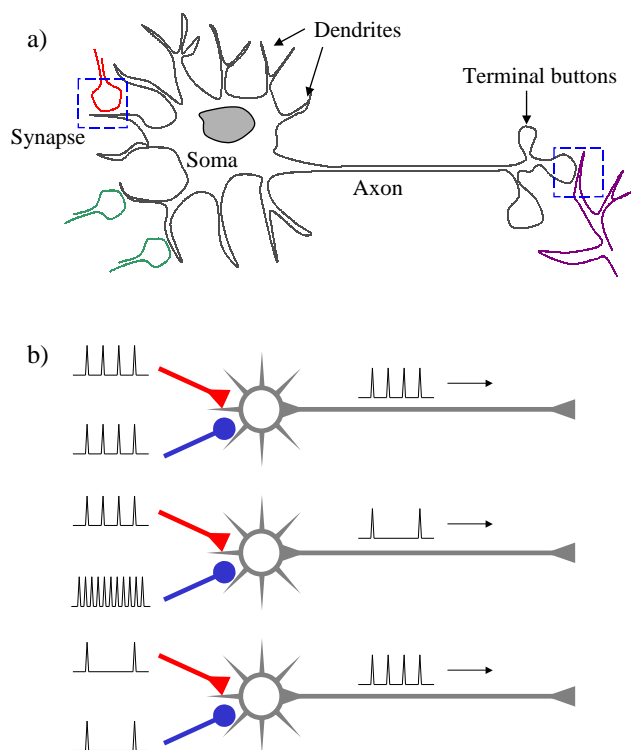


Figure 7.1. Structural and functional features of a nerve cell. Afferent (=incoming) synapses mainly connect to the soma and dendrites (although some are also found on the axon). The axon may greatly vary in length (from millimeters to a meter and more). The terminal buttons form the efferent synapse by which the neuron will control other neurons in turn. b: The afferent synapses may be either excitatory (red cones) or inhibitory (blue circles), and the balance of their respective firing rates will determine the efferent activity of the neuron in turn.

¹For which there are specialized sensory cells, just as there are for sound and light – but we have no awareness of their perceptions.

ferent synapses, where they will trigger the release of neurotransmitters from the terminal buttons. In this way, they will influence the activity of the next nerve or other excitable cell. All action potentials will be of similar amplitude and duration; it is the repetition rate that represents the level of activity (Figure 7.1b).

In all synapses, the presynaptic cell will always be a neuron². Postsynaptic cells can be either neurons, striated or smooth muscle cells, or gland cells (Figure 7.2a). In the case of skeletal muscle, the presynaptic neuron will be part of the somatic nervous system. In contrast, neurons that project to the heart muscle will be part of the autonomic system – none of us can voluntarily change the heartbeat. While in many synapses the presynaptic and postsynaptic membranes are in close apposition, thus ensuring rapid action, this is not necessarily the case in the effector synapses of the autonomic nervous system, which frequently do not have extremely time-critical missions.

Figure 7.2b shows an electron microscopic picture of a neuromuscular synapse. You can see the very narrow synaptic cleft, the numerous synaptic vesicles, and the actin / myosin filaments in the postsynaptic muscle cell. No space is wasted in this very fast and efficient synapse. Figure 7.2c shows a lower power (light microscopic) preparation of a nerve ending that branches toward multiple muscle cells. The synaptic buttons are clearly visible. Note that a single nerve cell controls several muscle fibers (up to several hundred, in fact). Synaptic excitation will trigger an all-out action potential at the muscle cell membrane that will be propagated swiftly along the entire expanse of the muscle fiber.

7.1. Structure and function of synapses

As we have seen, the presynaptic action potential will open voltage-gated calcium channels and thereby trigger exocytosis of the neurotransmitter that is stored in vesicles. The transmitter will then bind to a postsynaptic receptor. This will typically result in a local change to the postsynaptic membrane potential, which may or may not trigger a complete action potential (Figure 7.3). While a great many different transmitters exist, individual neurons only seem to be using very few different ones. Although the time-honoured textbook dogma of one transmitter per neuron only is no longer valid, we will, for the purpose of this class, pretend it to be.

Calcium promotes transmitter exocytosis at multiple stages. There are several pools of neurotransmitter vesicles in the nerve ending, which differ by their maturity (e.g., amount of transmitter stored) and their availability for immediate exocytosis. One effect of calcium consists in the recruit-

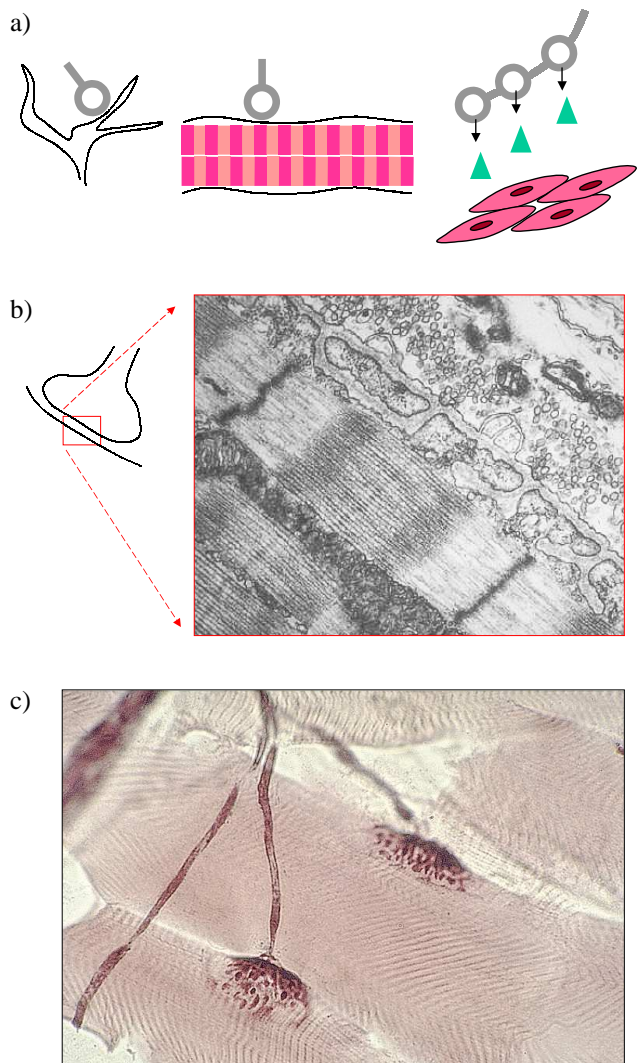


Figure 7.2. Structures of synapses. a: Some anatomical variations. Left: An interneuronal synapse connects two nerve cells. Middle: A neuromuscular synapse (also called motor endplate) connects the terminal button of a motoneuron to a skeletal muscle cell. Right: An autonomic nerve cell may form several secretory varicosities that release the transmitter in the target tissue, without forming a circumscribed contact zone with an individual target cell. b: Electron microscopy of a neuromuscular synapse. The synaptic cleft is very narrow, so that diffusion of secreted transmitter to the receptors on the postsynaptic membrane is fast. Numerous vesicles ‘line up’ close to the presynaptic membrane. Note the striated myofilament in the postsynaptic cell. c: Light microscopy. A single neuron branches out and sends terminal buttons to multiple muscle fibers.

ment of vesicles from an immobile, cell skeleton-attached pool into the mobile pool. This is achieved by calmodulin-dependent phosphorylation of synapsin, a protein associated with the cytosolic surface of the vesicle membrane. Mobilized vesicles will attach themselves to the cytoplasmic membrane. Attachment is mediated by mutual recognition of so-called SNARE membrane proteins located on both the vesicle and the cytoplasmic membrane. One such pro-

²Or a sensory cell, which are in fact specialized neurons.

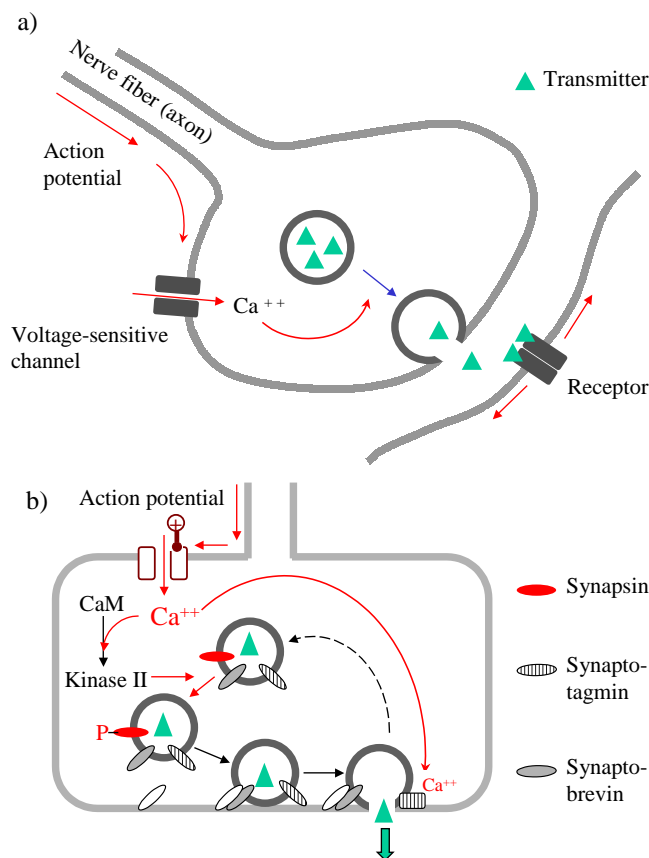


Figure 7.3. Mechanism of transmitter release. a: The presynaptic action potential opens voltage-gated Ca^{++} channels. Ca^{++} triggers exocytosis of neurotransmitters stored in presynaptic vesicles. b: Some proteins (out of many more) that are involved in exocytosis. Ca^{++} is involved at multiple stages. By binding to calmodulin (CaM), it promotes phosphorylation of synapsin, which 'primes' the transmitter vesicle but does not immediately lead to exocytosis. Adhesion of primed vesicles to the presynaptic membrane is mediated by synaptobrevin and other SNARE proteins. Synaptotagmin is activated directly by Ca^{++} and participates in the final step of secretion.

tein - synaptobrevin - is the target of tetanus and botulinum toxins³.

The very fast release of neurotransmitter (1 millisecond after presynaptic excitation) is due to the exocytosis from pre-attached vesicles. This is effected via synaptotagmin, which binds and directly responds to calcium. Besides the proteins mentioned, there is a somewhat distressing multitude of additional proteins also participating in neurotrans-

mitter release. The precise roles of most of these proteins remain unsettled.

7.2. Mechanisms of drug action on synapses

Drugs may influence synaptic transmission by different mechanisms:

1. Direct agonists and antagonists will directly bind to the postsynaptic receptor and either activate or competitively block it.
2. In many synapses, there are both post- and presynaptic receptors for the transmitter. The transmitter will act in an inhibitory fashion at the presynaptic membrane, thus providing for feedback control of release (Figure 7.4a). If its action is excitatory at the postsynaptic membrane, it is easy to see that the two receptors should be different. In this case, drugs may be developed that act solely on the presynaptic receptor. Such presynaptic agonists will then reduce the amount of transmitter available at the postsynaptic membrane, whereas presynaptic antagonists will increase it.
3. Drugs may augment the effect of the endogenous neurotransmitter by inhibiting its usually very rapid elimination from the synaptic cleft by either enzymatic degradation or presynaptic reuptake (Figure 7.4b). Enzymatic degradation occurs with acetylcholine, whereas presynaptic reuptake is used to scavenge most other small transmitter molecules.
4. Within the presynaptic neuron, synthesis, breakdown, or vesicular accumulation of the transmitters may be altered. This may lead to short-term release and long-term depletion of neurotransmitter. These mechanisms are relevant to cocaine and mechanistically similar drugs of abuse. Drugs that act according to any of these mechanisms are usually referred to as indirect agonists or antagonists, respectively.

We will see examples of all of these mechanisms in subsequent chapters.

7.3. Pharmacologically important neurotransmitters and their receptors

Some examples of important neurotransmitters are shown in Figure 7.5. The first one to be recognized – and still one of those most important for pharmacotherapy – is acetylcholine. Its enzymatic cleavage by cholinesterase enables its extremely fast inactivation, which is necessary in a synapse with very high repetition rates such as the neuro-muscular junction in skeletal muscle. However, it is also used in slower synapses in the peripheral autonomic nervous system, as well as in the central nervous system;

³Either toxin consists of two chains – a heavy chain, which targets the toxin to specific nerve cells, and a light chain, which is a protease with specificity for synaptobrevin and is actually interchangeable. Cleavage of synaptobrevin will disrupt transmitter exocytosis. The radically different consequences – flaccid paralysis with botulinum toxin, violent spasms with tetanus toxin – are due to the different neurons targeted: inhibitory neurons in the latter case, excitatory neurons in the former.

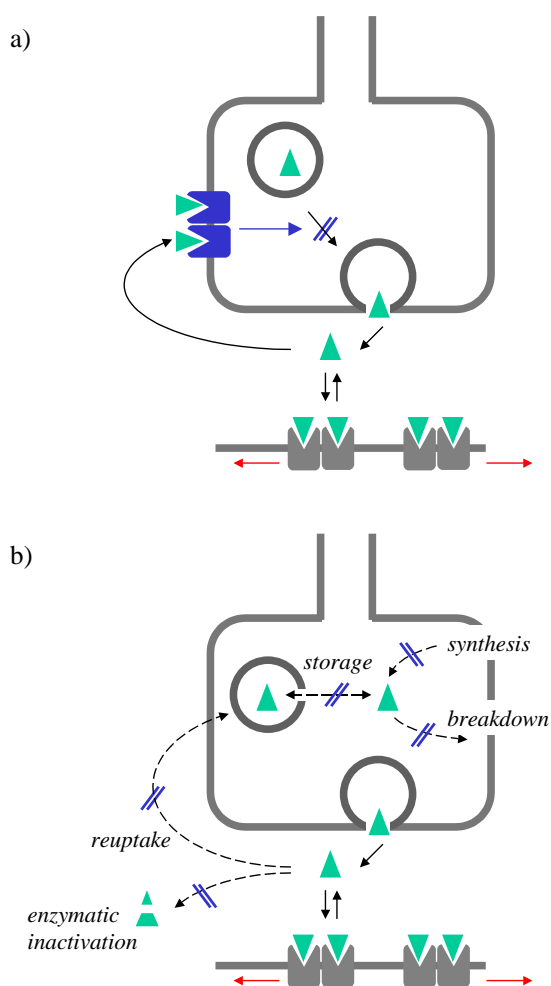


Figure 7.4. Mechanisms of action of indirect agonists or antagonists. a: Presynaptic receptors mediate negative feedback of transmitter release. Agonists at presynaptic receptors will behave as indirect antagonists (of postsynaptic activity, that is). b: There are many different potential mechanisms of drugs to increase or decrease the availability of transmitter.

we will see later that these different types of cholinergic synapses may be targeted by specific drugs.

Norepinephrine occurs in the peripheral and central autonomic system. The most important problem that is addressed by drugs acting on adrenergic synapses is high blood pressure. Many of these drugs act as postsynaptic antagonists or as presynaptic agonists, respectively. Dopamine differs from norepinephrine only by lacking its single aliphatic hydroxyl group. Parkinson's disease is due to a dopamine deficit owing to the degeneration of a fairly tiny group of nerve cells in the brainstem (called the 'substantia nigra', black substance). Application of L-DOPA, the precursor of dopamine, is the mainstay of therapy.

While both dopamine and norepinephrine are derived from tyrosine, serotonin (5-hydroxytryptamine, 5-HT) is derived from tryptophan. All three transmitters are very important

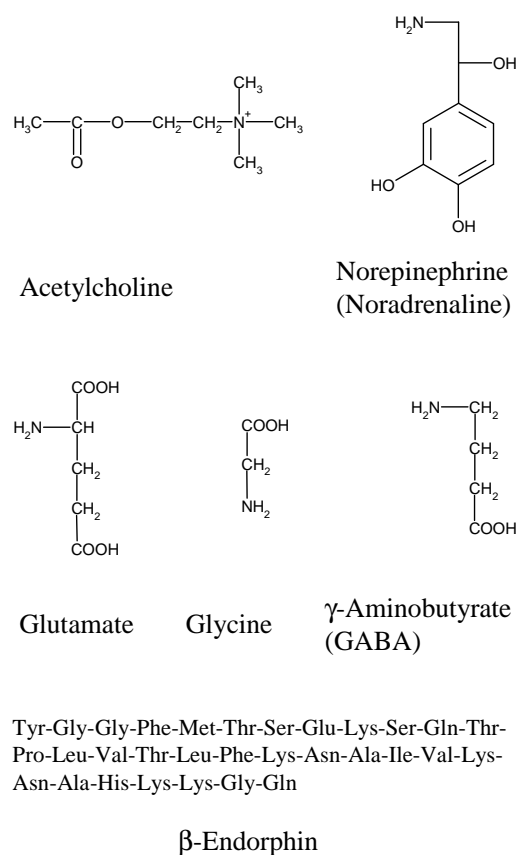


Figure 7.5. Structures of some neurotransmitters. Receptors for all of these are targets of drugs in current use.

in psychiatric disorders, and accordingly many psychopharmacological drugs act on their receptors.

Glutamate is the most widespread transmitter in the central nervous system. Despite its widespread occurrence, there are few clinically useful drugs that target glutamatergic synapses. In some places in the brain, there is an antagonism between dopaminergic and glutamatergic neurons. Accordingly, inhibitors of glutamate receptors are used in addition to L-DOPA in the treatment of Parkinson's disease. An example is amantadine, which acts on the NMDA type glutamate receptor (see next section).

GABA (γ-aminobutyric acid) is derived from glutamate by decarboxylation. It occurs predominantly in inhibitory synapses in the central nervous system, as does glycine. Tetanus toxin acts on glycinergic neurons in the spinal cord, which exert inhibitory regulation on the α-motoneurons⁴.

β-Endorphin is but one of an ever-increasing number of peptide neurotransmitters, and one of the rare cases in which there actually are specific agonists and antagonists – the name endorphin stands for 'endogenous morphine'.

⁴The nerve cells that sit in the spinal cord and relay the instructions for voluntary movements to the skeletal muscle. The axons of these cells reach all the way to the muscle cells and form the neuromuscular synapses discussed above.

Morphine and other opioids act on receptors for β -endorphin and the related enkephalins. β -Endorphin was found earlier than the other peptide transmitters because opioids were made available by nature; they could be used as probes to find the receptors, which could then in turn be used to screen brain extracts for substances that would displace opioids from their receptors.

7.4. Neurotransmitter receptors

The receptors for neurotransmitters fall into two broad classes:

- Ligand-gated ion channels, and
- G protein-coupled receptors (GPCR).

Ligand-gated channels will respond to the binding of a specific ligand with either opening or closing. We have seen an example of a ligand-gated channel before – the K_{ir} /sulfonyleurea system. There, the physiological agonist (ATP) acted from within the cell and effected channel closure. With neurotransmitter receptor channels, the agonists act from outside the cell and will cause channel opening.

Ligand-gated channels (and their ion selectivities) include:

- Nicotinic acetylcholine receptor (Na^+ , K^+ , Ca^{++})
- NMDA glutamate receptor (Na^+ , K^+ , Ca^{++})
- ADP (Purine) receptor P2X (Na^+ , K^+ , Ca^{++})
- Serotonin receptor 5-HT₃ (Na^+ , K^+ , Ca^{++})
- Serotonin receptor MOD₁ (Cl^-)
- Glycine receptor (Cl^-)
- GABA_A receptor (Cl^-)

Despite their different agonist and ion selectivities, all the above receptors belong to one homologous family and share the same overall structure. We will look in some detail at the workings of the nicotinic acetylcholine receptor in a subsequent chapter.

So what is the ‘nicotinic’ acetylcholine receptor, and what is the ‘NMDA’ glutamate receptor? These names reflect those of non-physiological agonists, nicotine and N-methyl-D-aspartate⁵, that selectively stimulate the receptors in question. Other types of receptors for acetylcholine or glutamate respond to different non-physiological agonists. As discussed previously, artificial agonists and agonists that exceed the natural agonists in receptor selectivity are important both for the experimental study of the receptors, and for applied pharmacotherapy.

The first four receptor channels listed above illustrate that the permeability of ligand-gated channels is not always very specific for one particular ion. In excitable cells, however, the major effect with these receptors will be due to the increased permeability for Na^+ , because this will offset the predominant effect of the K^+ permeability on the resting potential. The effect of Ca^{++} permeability on the membrane potential will not be quite as great as that of Na^+ , because its concentration is much lower (cf. the Goldman equation). However, because the intracellular Ca^{++} concentration is normally very low, the Ca^{++} influx will suffice to increase it significantly. The Ca^{++} influx therefore constitutes a biochemical signal that occurs simultaneously with the action potential.

The ‘prototype’ (i.e., the most-studied example) of the ligand-gated synaptic channel is the nicotinic acetylcholine receptor; we will look at its workings in more detail in a later chapter. The other receptors listed are all structurally homologous to the nicotinic acetylcholine receptor and presumably work in much the same way. Interestingly, the NMDA receptor has binding sites for two different ligands – one for glutamate, and another one for glycine, which are located on separate subunits⁶ of the pentameric channel.

G protein-coupled receptors (GPCR) constitute the largest structural class of hormone and neurotransmitter receptors. They are sometimes also referred to as ‘metabotropic’ receptors, since they do not immediately affect the membrane potential but instead trigger biochemical cascades (which then, at a later stage, may yet alter the membrane potential, through ligand-gated channels that have intracellularly located binding sites for G proteins or second messengers). Examples are:

- Muscarinic acetylcholine receptors (several types)
- Catecholamine receptors (several types)
- Serotonin receptors 5-HT_{1,2,4,6}
- GABA_B receptors
- ‘Metabotropic’ glutamate receptors (11 subtypes)
- Purine receptors (P2Y): Adenosine, AMP, ADP, ATP
- Peptide hormone receptors

Several things are of note here:

1. The vague phrase ‘peptide receptors’ hides the fact that the number of peptide neurotransmitters (oxytocin, endorphin, cholecystokinin, galanin, ...) is actually much larger than that of small molecules (comprising amino acids, amines, and acetylcholine). However, for most of these receptors, no specific agonists or antagonists are

⁵This receptor is frequently referred to simply as the NMDA receptor; however, NMDA is not a physiological transmitter.

⁶Another, homologous receptor that has more recently been characterized is sensitive to glycine only yet shares the conductivity for cations, so that glycine turns out not to be a purely inhibitory transmitter after all.

available, and they are therefore not presently in the focus of pharmacotherapy to the same extent as the receptors for small molecular transmitters⁷.

2. Purine receptors: Adenosine, AMP, ADP and ATP can act extracellularly as hormones or transmitters, too! In particular, adenosine receptors that occur in the heart, the brain, and the lung are the targets of theophylline and caffeine. Adenosine itself is being used for the treatment of a special type of cardiac arrhythmia.
3. Several transmitters – acetylcholine, glutamate, serotonin, GABA, and adenosine and its various phosphates – have receptors both among the ligand-gated channels and the G protein-coupled receptors. Thus, these transmitters evidently serve multiple purposes in the nervous system.
4. Even among the G protein-coupled receptors responding to one (hormone or) transmitter, there is considerable variety, and the downstream signaling mechanisms employed by these various receptors may vary. E.g., among the 11 GPCR for glutamate, at least three downstream signaling mechanisms are represented.

The various signalling mechanisms triggered by stimulation of GPCR will be discussed in more detail in a subsequent chapter.

7.5. Overview of the autonomic nervous system

It was stated at the beginning that the peripheral autonomic system has a prominent place as a site of drug action. We will now look at the organization of this system, and at the distribution of transmitter receptors within it. This will enable us to understand the effects of drugs acting upon this system and rationales behind their usage.

The autonomic nervous system consists of two functionally distinct parts that frequently exert antagonistic effects on their target organs. These are referred to as the sympathetic and the parasympathetic system, respectively. Figure 7.6 depicts some essential features. The parasympathetic system, for the most part, emerges from the central nervous system at the level of the *medulla oblongata*, which is the lowermost part of the brain⁸. These neurons reach

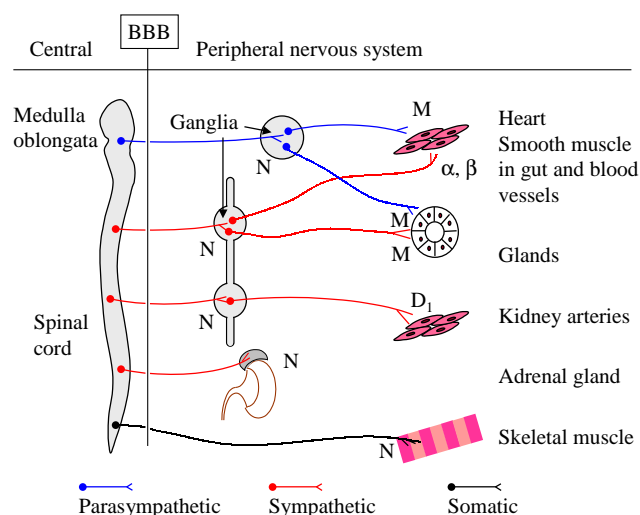


Figure 7.6. Organisation of the autonomic nervous system, and the chemical types of synapses found within it. M: Muscarinic, N: Nicotinic cholinergic receptors; D: Dopaminergic, α , β : Adrenergic receptors. (The innervation of skeletal muscles by α -motoneurons is shown for comparison but not part of the autonomic system.) BBB: Blood brain barrier. It protects the entire central nervous system, i.e. both the brain and the spinal cord.

some nerve centers in the periphery, which are named ganglia (singular: ganglion), where they trigger activity in secondary neurons that in turn reach out to the target organs. The sympathetic system mostly emerges at the thoracic portion of the spinal cord. It too has relay neurons in peripheral ganglia (which are connected with each other in the so-called ‘sympathetic chains’, located on either side of the spine). The parasympathetic and sympathetic ganglia are outside the central nervous system, and therefore readily accessible to drugs that do not cross the blood brain barrier.

The target tissues that are controlled by the secondary neurons (the ones originating in the ganglia) include:

- Secretory cells in various glands, both exocrine and endocrine;
- Heart conduction system and muscle cells;
- Smooth muscle cells in the intestine, other hollow organs (bronchi, urinary tract, sexual organs, etc.) and in the blood vessels.

Figure 7.6 also shows the major types of neurotransmitter receptors found within the autonomic nervous system:

- The nicotinic acetylcholine receptor occurs in both the sympathetic and the parasympathetic ganglia. The receptors found in the neuromuscular synapse⁹ are of the

⁷Many of these peptides are found in the brain as well as in the peripheral autonomic system, e.g. cholecystokinin and gastrin. The role in the peripheral organs can be studied with isolated organs and the peptide ligands themselves (cholecystokinin induces gall bladder secretion, gastrin induces gastric HCl secretion...). However, in contrast to small transmitters such as norepinephrine or acetylcholine, the peptides are not useful starting points for the development of specific agonists or antagonists that would be small and stable enough to cross the blood brain barrier; therefore, the development of drugs acting on these receptors is lagging behind.

⁸The higher parts of the brain have been disregarded, which I consider

to be in accord with general UW practice.

⁹Within the skeletal muscle. These neurons – the α -motoneurons – are not part of the autonomic but of the somatic system.

nicotinic type as well. However, the subtype is different, and therefore selective drug action is possible.

- Muscarinic acetylcholine receptors occur in the target tissues. They are mostly found in parasympathetic synapses, but they also occur in the sympathetically innervated sweat glands.
- Adrenergic receptors are always related to sympathetic activity, either within synapses (as shown here), or diffusely distributed and by responding to circulating epinephrine.
- Dopamine D_1 receptors are less widespread than adrenergic receptors. One prominent occurrence is in the kidney arteries. Accordingly, dopamine and related agonists are being used in intensive care treatment of acute kidney failure to improve kidney perfusion.

Very commonly, a target tissue will be stimulated by the sympathetic system and inhibited by the parasympathetic system, or vice versa. Examples are found in table 7.1. Among the parasympathetic responses listed there, we find stimulation of smooth muscle in the bronchi, and relaxation of smooth muscle in the arterioles; both are mediated by muscarinic acetylcholine receptors (cf. Figure 7.6). Here, we have an example of diverse effector mechanisms triggered from similar receptors. Similarly, the adrenergic receptors can operate different intracellular switches as needed. These different effector mechanisms are covered in some more detail in the chapter on G protein-coupled receptors.

A ‘take-home’ message from table 7.1 is that, by and large, muscarinic receptors mediate the parasympathetic effects, whereas the sympathetic ones are mediated by adrenergic receptors.

From the effects of the autonomic nervous system on the various target organs (table 7.1), we can easily understand several applications of drugs that cause synaptic stimulation or inhibition:

- In patients having undergone abdominal surgery, quite frequently the activity of the intestine is sluggish. Drugs that stimulate muscarinic receptors will help to correct this.
- As we have seen, drugs that block α -adrenergic receptors (e.g., phenoxybenzamine) will help to lower the resistance in arterioles and therefore reduce blood pressure.
- Blockers of β_1 -adrenoceptors help to reduce the workload of the heart, but they sometimes slow down the generation or propagation of excitation too much, resulting in slow and occasionally irregular heartbeat.
- Drugs that stimulate β_2 -adrenoceptors will help to dilate the bronchi (by reducing the smooth muscle tone

there) will be useful in asthma, which basically consists in impeded air flow due to a spastic narrowing of the bronchi.

- If the effect of β_2 agonists in asthma proves insufficient, one additional therapeutic option is to add a drug that will inhibit the cholinergic (parasympathetic) stimulation of the bronchial smooth muscle, such as ipratropium bromide¹⁰.

A peculiar element within the autonomic nervous system is the medulla (inner part) of the adrenal gland. This is the site of production for epinephrine and norepinephrine that are released into the circulation. It is directly controlled by cholinergic neurons emerging from the spinal cord, so it assumes the place of a sympathetic ganglion. In fact, the cells in the adrenal medulla are of neural origin – they are nerve cells turned gland cells. In contrast, the cortex (outer part) of the adrenal gland) is a ‘proper’ gland tissue not of neural but mesodermal origin. The endocrine (hormonal) and the neural system are not as cleanly separated as our neat abstractions suggest.¹¹

Table 7.1 also lists the effects of sympathetic and parasympathetic stimuli on the pupil of the eye pupil (this had been omitted from Figure 7.6, which is incomplete in many ways). In the case of the pupil, the antagonism between sympathetic and parasympathetic system is due not to antagonistic innervation of the same target cells but of two antagonistic muscles, the dilatator and the sphincter muscles of the iris, respectively (Figure 7.7). While the autonomic control of the iris is not overwhelmingly important in applied pharmacotherapy¹², it is a very useful diagnostic

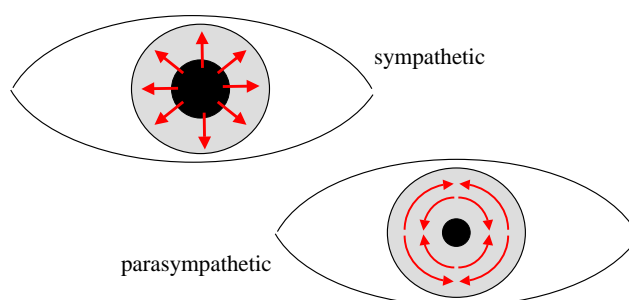


Figure 7.7. Response of the pupil to activation of autonomous innervation. The movements are caused by the radial and the concentric muscle fibers of the pupil dilatator and sphincter muscles, which have sympathetic/adrenergic and parasympathetic/cholinergic innervation and receptors, respectively.

¹⁰ Other options are concerned with the suppression of the allergic / inflammatory process that trigger the bronchial constriction in the first place.

¹¹ Another case in point is the hypophyseal gland, which like the adrenal gland combines endodermal and neural gland cells in one organ; both cell types secrete peptide hormones into the circulation.

¹² Drugs that widen the pupil (e.g., atropine) are, however, used by oph-

| Organ | Part / tissue | Parasympathetic impulse response (receptors) | Sympathetic impulse response (receptors) |
|---------------|--------------------------------|--|--|
| Eye | Radial muscle of iris | – | Contraction (widened pupil; α -adrenergic) |
| | Sphincter muscle of iris | Contraction (narrow pupil; muscarinic) | – |
| Heart | Conduction system | Slower rhythm and conduction (muscarinic) | Faster rhythm and conduction (β_1 ; largely due to circulating epinephrine) |
| | Heart muscle | Reduced force of contraction (muscarinic) | Increased force of contraction (β_1 -adrenergic) |
| Blood vessels | Arterioles | Dilatation (muscarinic) | Skin, intestine: Contraction (α_1) |
| | | | Skeletal muscle: Dilatation (β) |
| | Kidney Arteries | (–) | Dilatation (dopamine D_1) |
| | Veins | Dilatation (muscarinic) | Contraction (α_1) |
| Lungs | Bronchial tree (smooth muscle) | Contraction (muscarinic) | Dilatation (β_2) |
| | Bronchial glands | Secretion (muscarinic) | – |
| Intestine | | Increased motility and tone (muscarinic) | Decreased motility and tone (α) |
| Sweat glands | Palms, some other locations | – | Secretion (muscarinic) |
| | Other | Secretion (muscarinic) | – |

Table 7.1. Examples of organ responses to autonomic innervation.

marker. E.g., in poisoning with drugs that induce or amplify cholinergic action we will see a pronounced narrowing of the pupil. This is called ‘miosis’ in doctors’ speak; widening (observed e.g. with cocaine) is ‘mydriasis’. One of the glorious things about medicine is the profusion of cryptic names for simple things.

thalmologists in diagnostic procedures. An additional effect of atropine is to relax the ciliary muscle, which modifies the shape of the lens to switch focus between far and near. Relaxation of the ciliary muscle, then, facilitates ‘objective’ measurement of the eyes’ refractive properties, undisturbed by the patients’ attempts to adjust the focus.

(Notes)

Chapter 8. G protein-coupled receptors

G protein-coupled receptors (GPCR) represent the largest group among the receptors of both hormones and neurotransmitters, and accordingly have a prominent role as drug targets. The percentage of all drugs in use today that act on one or the other GPCR is given in the literature as 25-50%, and it is likely to increase in the future¹. Examples are:

- Muscarinic acetylcholine receptors (several types)
- Catecholamine receptors
- Serotonin receptors 5-HT_{1,2,4,6}
- GABA_B receptor
- 'Metabotropic' glutamate receptors (11 subtypes)
- Purine receptors (P2Y): Adenosine, AMP, ADP, ATP
- Peptide hormone receptors

As noted before, most of the presently used drugs target receptors of small hormone and transmitter molecules, to which they are structurally related. The number of drugs that target receptors for peptide mediators is very limited. Notable examples are the opioids (derivatives and analogs of morphine) and the angiotensin receptor blocker valsartan (cf. the introductory chapter). However, peptide receptors are very much in the focus of current drug development efforts, and it seems likely that in the future a substantial number of new drugs that target currently inaccessible receptors will become available.

8.1. Structure and function of G protein-coupled receptors

G protein-coupled receptors all belong to one structural family, which is frequently referred to as the '7-TM' receptor family. This name refers to the 7 α -helical transmembrane domains, which occur in all of these molecules. Variability is larger in the N-terminal and C-terminal parts and the loops intervening between the transmembrane domains, which are exposed to the extracellular and the cytoplasmic spaces, respectively.

The basic mode of action of a G protein coupled receptor and the G protein activated by it is illustrated in Figure 8.1. Binding of the agonist to the extracellular face of the receptor triggers a conformational change that is communicated

¹The reason for this variation may be inclusion or exclusion of anti-bacterial drugs (antibiotics), which don't have a drug target in human physiology.

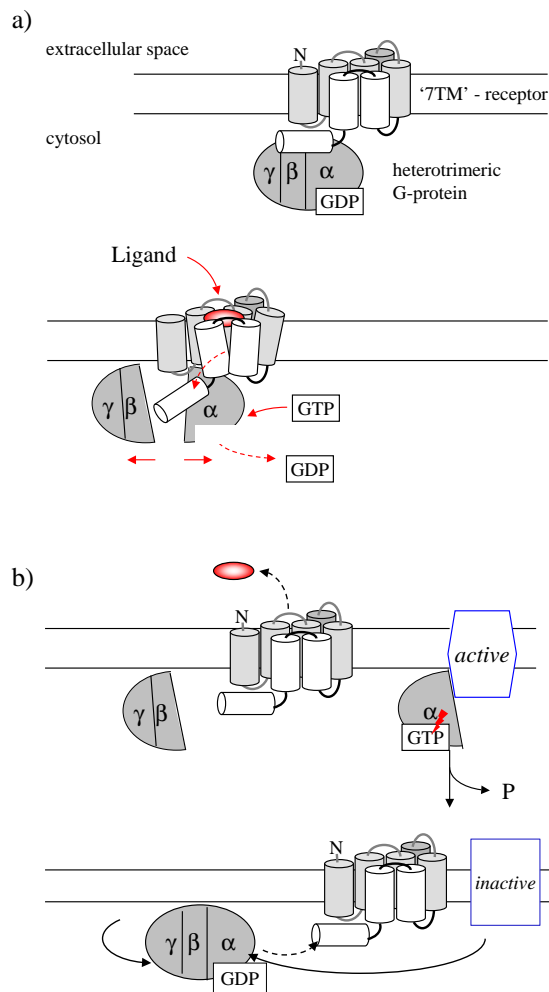


Figure 8.1. Function of G protein-coupled receptors. a: The receptor has seven transmembrane helices ('7-TM'). Intracellular parts of the receptor molecule are associated with a heterotrimeric G protein that in the resting state has GDP bound to it. Upon ligand binding, the receptor undergoes a conformational change that is transmitted to the G-protein attached to it. This causes the α subunit to exchange GDP for GTP and then dissociate from the $\beta\gamma$ dimer. b: The α subunit seeks its target protein, the activity of which it modulates (here: increases). The α subunit has a built-in GTPase activity. Upon cleavage of GTP to GDP, it leaves the effector and reassociates with the $\beta\gamma$ dimer, thus expiring the receptor signal. The G protein then associates with a GPCR again.

to the intracellular portion of the receptor and there is relayed to the G protein. The latter is a trimer, comprised of one α , β and γ subunit each. The β and γ subunits remain associated throughout the functional cycle of the G protein. Interaction with the receptor involves all three subunits of the G protein. When the receptor is activated, it will transmit its conformational change to G protein. This will trig-

ger the α subunit to exchange its associated GDP molecule (a leftover from the last round of activation) for GTP, and to dissociate from both the $\beta\gamma$ -dimer and the receptor. It will then find and bind to its effector molecule, which will result in either activation or inhibition of the effector.

The signal is expired through the intrinsic GTPase activity of the α subunit, which after some random² time interval will cleave the GTP to GDP. This will cause the α subunit to fall back into its inactive conformation and leave the effector, which in turn will resume its previous functional state. It will then re-associate with the $\beta\gamma$ dimer to complete the cycle and wait for the next round of activation event by the same or another receptor molecule.

In Figure 8.1a, the effector was shown to be activated by the G-protein. An example of this is the stimulation of adenylate cyclase, which occurs upon stimulation e.g. of β -adrenergic receptors and is mediated by the stimulatory G protein (G_s ; Figure 8.2a). Other major effector mechanisms include:

- The inhibition of adenylate cyclase. This is mediated by the inhibitory G protein (G_i) α subunit. Triggers for this response include the adrenergic α_2 -receptor, which is responsible for presynaptic feedback inhibition in adrenergic synapses, and the muscarinic M_2 receptor (Figure 8.2b).
- Inhibition or activation of phosphodiesterases, in particular cGMP-specific ones. cGMP (cyclic guanosine monophosphate) is an intracellular second messenger similar to cAMP. Besides activating a group of protein kinases³, cGMP acts directly on several ion channels. In the rods and cones (visual sensory cells) of the retina, rhodopsin (a particular type of GPCR, activated by light-induced structural change to its ligand retinal) activates phosphodiesterase, which in turn depletes cGMP and thus triggers the inactivation of a cGMP-dependent Na^+ channel. This is not of immediate relevance in pharmacology, but it illustrates that G protein-mediated responses can be very fast indeed.
- Activation of phospholipase C β (Figure 8.2c). This enzyme is associated with the inner surface of the plasma membrane and splits the phospholipid phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Diacylglycerol is hydrophobic and remains associated with the membrane, where it will activate protein kinase C, which in turn will activate a broad and cell-dependent spectrum of target proteins. IP_3 is water-soluble and

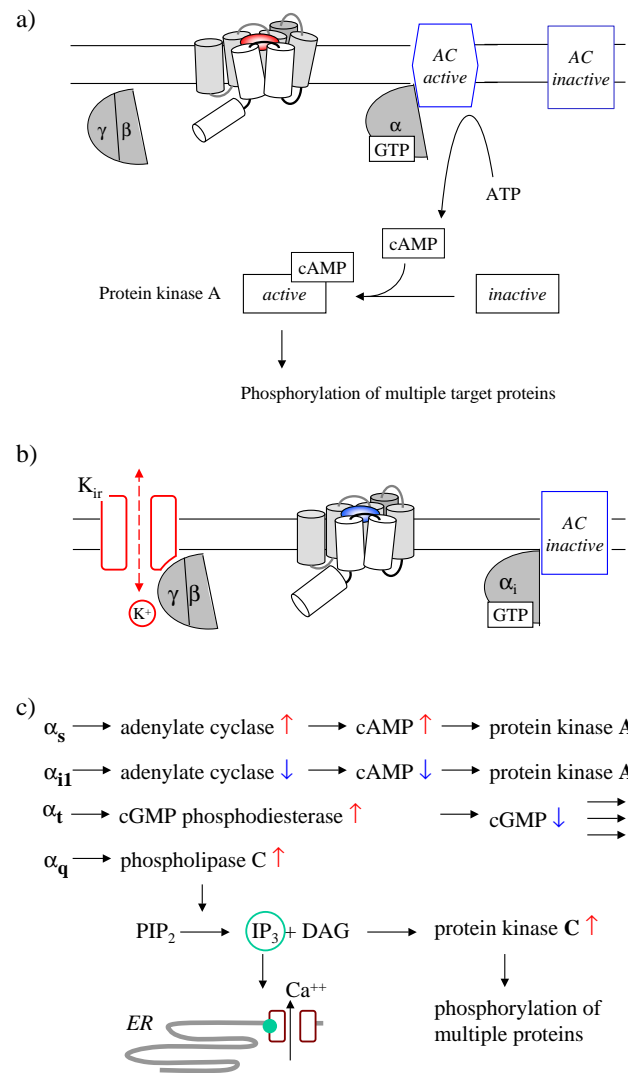


Figure 8.2. Examples of G protein signalling mechanisms. a: β -adrenergic receptors are coupled to the stimulatory protein (G_s) which activates adenylate cyclase. b: Muscarinic M_2 receptors activate the inhibitory G protein (G_i) which in turn inhibits adenylate cyclase. The $\beta\gamma$ -dimer of G_i also has regulatory activity: it opens a K_{ir} potassium channel. c: Overview of major signaling cascades triggered by different G_α subunits.

diffuses across the cytosol to the membrane of the endoplasmic reticulum, where it will activate a specific ligand-gated calcium channel. Therefore, it will raise the cytosolic calcium level (recall that the concentration of Ca^{++} is higher in the ER than in the cytosol). This is the major effector mechanism by which transmitters, hormones and drugs will promote contraction in the smooth muscle (remember that calcium will induce phosphorylation of the myosin light chain by myosin light chain kinase). Receptors that trigger this cascade include

- The α_1 -adrenergic receptor, found predominantly in blood vessels,

²Random for the individual molecule, but with a defined decay kinetics for large numbers.

³Some of which again affect smooth muscle tone; this will be covered in more detail in the chapter on nitric oxide.

- Various Muscarinic acetylcholine receptors, found e.g. in the intestinal smooth muscle,
- The oxytocin receptor, found in smooth muscle cells in the uterus, where it triggers labour.

However, the phospholipase C cascade is not restricted to smooth muscle cells but is ubiquitous.

- Activation of phospholipase A₂, which releases arachidonic acid and thus initiates synthesis of prostaglandins and related eicosanoid mediators. Again, we will see more about this in a dedicated chapter.
- Opening or closing of ion channels. In this way, the ‘metabotropic’ receptors may change the membrane potential as well.

While the more numerous and clear-cut regulatory activities of G proteins are associated with α subunits, the $\beta\gamma$ dimers may also influence some downstream effector. An example is the effect of the muscarinic M₂ receptor on the activity of a potassium channel, which, like the one associated with the sulfonylurea receptor, belongs to the ‘inward rectifier’ class. The channel opening effected by the $\beta\gamma$ dimer will hyperpolarize the membrane and reduce its responsiveness to excitation.

Table 8.1 lists several transmitters, and their cognate receptor and signalling mechanisms. All three of the effector mechanisms listed in table 8.1 for the adrenergic receptors also occur among the ‘metabotropic’ glutamate receptors.

8.2. The complexity of G protein signalling

Although it is usually pointless to point out the complexity of biological systems, here it may be appropriate.

| Agonist | Receptor(s) | Effector mechanism |
|-----------------------------|--|-------------------------|
| Norepinephrine, epinephrine | α_1 | IP ₃ + DAG ↑ |
| | α_2 | cAMP ↓ |
| | β_{1-3} | cAMP ↑ |
| Dopamine | D ₁ , D ₃ | cAMP ↑ |
| | D ₂ , D ₄ | cAMP ↓ |
| Acetylcholine | M ₁ , M ₄ , M ₅ | IP ₃ + DAG ↑ |
| | M ₂ , M ₃ | cAMP ↓ |

Table 8.1. Signaling mechanisms triggered by pharmacologically important agonists and their respective subtypes of G protein-coupled receptors.

G protein coupled receptors, besides their roles in responding to intrinsic signals (hormones and transmitters), are also responsible for our ability to smell and taste, which means that G protein-coupled receptors respond to extrinsic rather than intrinsic signals. Beyond the large number of receptors with known roles, there is an even larger number of so-called ‘orphan’ receptors, the ligands and functional roles of which have not yet been determined. The overall number of GPCR genes in the human genome is at least 300-400 – which corresponds to about 1% of all genes. As pointed out above, the significance of G protein-coupled receptors in pharmacology is already great today, and it is likely to increase as more information on the ligands and functional roles of individual receptors will become available, and the interaction of drugs and receptors will be elucidated in structural detail.

G protein-coupled receptors function as adapters between the virtually boundless multitude and variety of extracellular hormone and neurotransmitter signals and the lower (yet still considerable) number of intracellular G-proteins. Therefore, it is very common to have receptors for multiple transmitters or hormones converge onto the same type of G protein and thus trigger the same response. E.g., glucagon and epinephrine both activate adenylate cyclase in the liver, through separate receptors but the very same G protein.

8.3. Agonist-specific coupling

On the other hand, receptors may be promiscuous as well and couple to more than one G protein. Many receptors appear to be pre-associated with G proteins in the absence of agonists. The pre-bound G protein may in turn change the conformation of the entire receptor and thus modify its affinity for mediator and drug molecules. If there is more than one type of G protein to couple to a given receptor, this may result in several sub-populations of the receptor that may exhibit diverging affinities for different agonists. This behaviour is known as ‘agonist-specific coupling’.

An example of agonist-specific coupling is shown in Figure 8.3. The receptor in question is the α_{1A} -adrenergic receptor. This receptor triggers at least two different intracellular signaling pathways: Arachidonic acid is released by phospholipase A₂, and inositoltriphosphate (IP₃) is released by phospholipase C. These two effects are mediated by two different G proteins that couple to the same receptor. If we compare the two parameters, the three compounds norepinephrine and meta- and para-octopamine yield substantially different potencies and efficacies. If we hadn’t been told otherwise, we would interpret this observation as evidence of two different receptors. In a sense, of course, different GPCR-G protein complexes *are* different receptors.

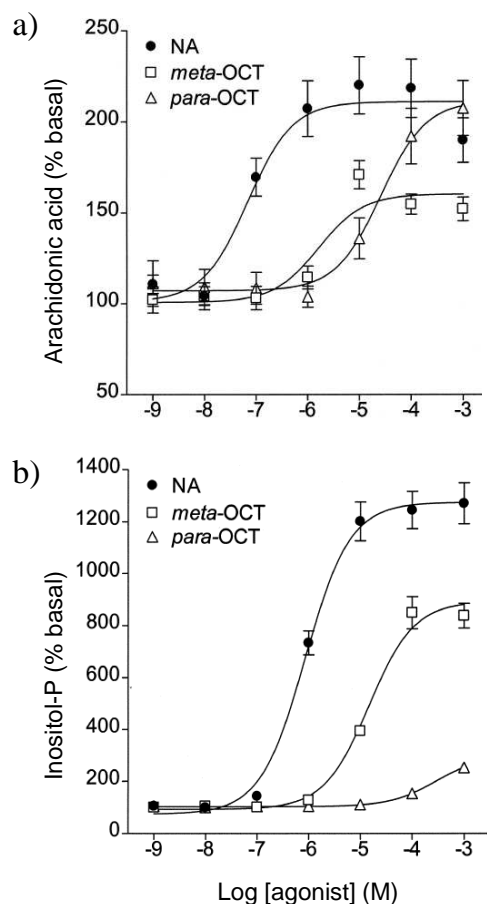


Figure 8.3. Agonist-specific coupling at α_{1A} -adrenergic receptors. The release of two different secondary messengers – arachidonic acid and inositoltrisphosphate – is triggered by two different G proteins. Norepinephrine (NA), meta-octopamine and para-octopamine all show different potencies and / or efficacies in the two different readouts, showing that the two G-proteins modify the interaction of the receptor with the agonist. Source: Naunyn Schmiedebergs Arch Pharmacol. 367:333-41 (2003).

8.4. GPCR oligomerization

Another source of variation is the oligomeric state of G protein-coupled receptors. In Figure 8.1, the receptor is depicted as a monomeric molecule, and indeed this was the prevailing model until fairly recently. However, it is now clear that very many GPCR are indeed oligomeric. This has several consequences:

- Efficacy and potency of a ligand may be different for monomeric and oligomeric receptors.
- Dose-effect curves may take different shapes, due to cooperative ligand binding⁴.
- Oligomers may be ‘homomers’ but also ‘heteromers’, which means that they may form from like or from dif-

ferent subunits. The existence of heteromers adds another dimension to the variability of receptor types, similar as with the voltage-gated potassium channels covered earlier.

- It is possible to develop multivalent drugs that bind to several binding sites simultaneously. This may result in very high affinity for the receptor, and it might allow the targeting of certain heteromeric subtypes, increasing the selectivity of drug action.

An example of a bivalent drug that binds to the M_1 muscarinic receptor is shown in Figure 8.4. The affinity of this molecule for the receptor is about 100 times higher than that of the conventional, monomeric agonist carbachol. Note that there is a spacer between the two ‘pharmacophores’, which will allow the latter to bind to two separate subunits of the receptor oligomer. The length of this spacer was systematically varied, and it was found that a number of three repeating subunits gave both the highest affinity and the strongest receptor activation. Molecules with shorter spacers still bound with considerable affinity but failed to stimulate the receptor, possibly because the sterical constraint imposed by the shorter spacer prevented the receptor from assuming its active conformation. Since GPCR are all structurally related, this finding suggests a surprisingly straightforward approach to the development of agonistic and antagonistic drugs from a single ‘lead compound’.

8.5. ‘Allosteric’ GPCR agonists and antagonists

While most drugs that act as agonists or agonists of GPCR appear to bind competitively, i.e. to the binding site of the physiological agonist, several compounds have been reported to bind to other sites, allowing them to bind simultaneously with the physiological agonist. Binding of such ‘allosteric’ effectors may promote or reduce activation by the physiological agonist without actually causing any effect in the absence of the latter. As an example, the interaction of two molecules with A_1 adenosine receptors⁵ is shown in Figure 8.5. While the ‘allosteric’ molecule in question (PD

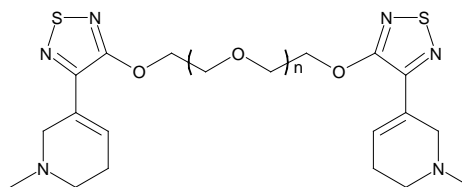


Figure 8.4. Structure of an experimental bivalent muscarinic M_1 agonist. Source: J. Med. Chem. 44:4563-4576 (2001).

⁴ This is completely analogous to the difference in oxygen binding between myoglobin and hemoglobin, and you may want to consult your biochemistry textbook for a refresher on the theory.

⁵ Adenosine receptors are targeted by drugs such as theophylline and caffeine.

81,723) does indeed enhance binding of the agonist (cyclohexyladenosine) at intermediate concentrations, it actually inhibits it at higher concentrations, which is not at all in keeping with the expectations for a true allosteric effector. An alternative interpretation is that the A_1 adenosine receptor is oligomeric, and that cyclohexyladenosine and PD 81,723 bind to the regular binding site but on separate subunits. If binding to separate subunits were cooperative, one would expect a pattern like the one observed here⁶.

(Notes)

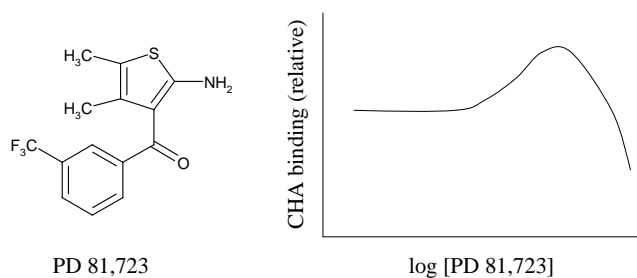


Figure 8.5. ‘Allosteric’ agonism on A_1 adenosine receptors by the experimental drug PD 81,723. Binding of the agonist cyclohexyladenosine (CHA) is increased by intermediate concentrations of PD 81,723 but inhibited by high concentrations. The latter feature is *not* typical of allosteric regulation. Source: Bruns, R.F. and Fergus, J.H., *Mol. Pharmacol* 38:939-949, 1990.

⁶The reported data predate the observation of GPCR oligomerization, so the authors are not to be faulted for their interpretation.

Chapter 9. Pharmacology of cholinergic synapses

As we have seen [in a previous chapter], acetylcholine occurs in synapses in both the somatic and the autonomic nervous system. The nicotinic acetylcholine receptor is found in the motor endplate of the skeletal muscle, and in both the sympathetic and the parasympathetic ganglia of the peripheral autonomic system. Muscarinic acetylcholine receptors are found at the endings of all secondary neurons within the parasympathetic part of the peripheral autonomous system. In addition, acetylcholine receptors of both types also occur in the brain. Drugs with a useful degree of selectivity for each of these targets are available and used in practical medicine. Selectivity is based on two principles:

1. Receptor type and subtype specificity of agonists or antagonists, and
2. Exclusion by the blood brain barrier of drugs intended for peripheral action.

One particular feature of cholinergic synapses is the mode of transmitter inactivation. While with most transmitters this is accomplished by presynaptic transmitter reuptake, acetylcholine is cleaved extracellularly by acetylcholinesterase. This enzyme is located on the postsynaptic membrane, right next to the receptors (Figure 9.1). Its activity is very high, so that the rate-limiting event in acetylcholine inactivation is not cleavage but dissociation from the receptor. Besides the direct receptor agonists and antagonists, blockers of cholinesterase (also referred to as ‘indirect agonists’) are an additional class of drugs that will modulate the effectiveness of transmission in cholinergic synapses.

9.1. Structure and function of the nicotinic acetylcholine receptor

The nicotinic acetylcholine receptor (NAR) is the most widely studied receptor ion channel. This is due to a very practical reason – availability. The receptor can be isolated in high yield from electric eel or electric ray, both of which use strong electric discharges to incapacitate their prey or for defence. In the electric organs of these fish, the receptor occurs in abundance in stacks of excitable cells (Figure 9.2). Importantly, however, the NAR and voltage-gated ion channels only occur on one side of the cell.

In the resting state, both sides of the cell will have the same membrane potential. As a consequence, the electric field vectors within the cell and within the entire stack will cancel each other out (Figure 9.2a). Electric stimulation will

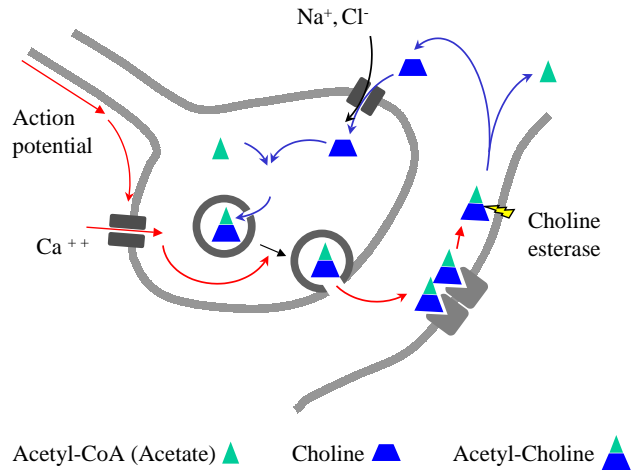


Figure 9.1. Overview of a cholinergic synapse. The transmitter is synthesized in the presynaptic terminal from choline and acetyl-CoA. Upon release, it binds to the postsynaptic receptor (of either the nicotinic or muscarinic type). Inactivation is by acetylcholinesterase, which is located at the postsynaptic membrane. Choline is taken up again into the presynaptic terminal by sodium and chloride cotransport.

depolarize one membrane in each cell and invert its electrical field. Now, all of a sudden, all field vectors in the entire stack will point into the same direction (Figure 9.2b), and thus will add up to one very strong electrical field. Each individual cell will contribute about 150 mV (the potential of its two membranes connected in series). Since the overall voltage is about 500-1000 V, this obviously requires several thousands of cells stacked on top of each other. Moreover, since both voltage and current are needed to make an impact on the prey, each level within the stack will need to have a sizeable surface area, thus providing for a large numbers of NAR molecules¹.

9.1.1. Overall structure

A variety of experimental approaches have been taken to study the structure of NAR. A very interesting one is ‘electron crystallography’, performed with NAR incorporated into artificial lipid membranes (liposomes) and there forming regularly packed arrays (Figure 9.3a, right). Electrons (not X rays, which are normally used in protein crystallography) scattered from these two-dimensional crystals will form a pattern that can be evaluated to yield a three-dimen-

¹The electric fish should be an excellent source of cholinesterase and voltage-gated channels, too, but I didn’t come across a report of its use for that purpose.

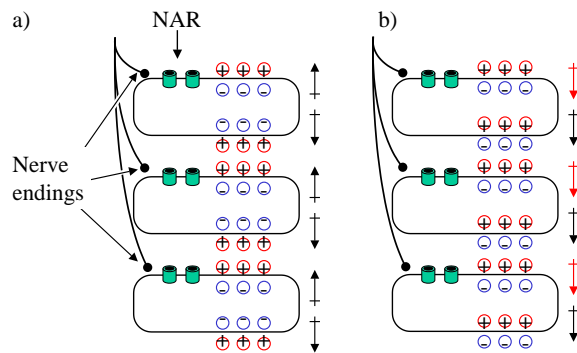


Figure 9.2. Sketch of a *Torpedo* electric organ. a: Several thousand disk-like cells are stacked up. Only one of the membranes is excitable by way of nicotinic acetylcholine receptors (NAR) and voltage-gated channels (not shown). In the resting state, the membrane potential is the same on both membranes in each cell, and the electric field vectors cancel out. b: Upon excitation, the top membrane of each cell is depolarized. Now, all electrical field vectors point into the same direction and add up to a total voltage of several hundred Volts.

sional map of the structure, provided that data are available from various tilt angles of the crystals relative to the electron beam. The resolution of structural images thus obtained is not quite the same as that of X-ray crystallography. However, X-ray data are often very hard to obtain with integral membrane proteins; in fact, in the structures available, the transmembrane parts are often missing².

Figure 9.3b shows ‘contour’ maps of the NAR, in top and in side view. In the top view, the identities of the individual subunits are also indicated; these have been determined using samples labelled with antibody fragments specific for each subunits. Note that the subunit composition of the NAR varies between different tissues. The $\alpha_2\beta\gamma\delta$ pattern shown here holds for the fish electric organs³, and for the muscle type NAR in humans. Neuronal NAR, including the one found in the autonomic ganglia, is $\alpha_2\beta_3$ or $\alpha_3\beta_2$. This structural difference accounts for the selective action of several agonistic and antagonistic drugs on muscles or the ganglia, respectively (see below).

In the side view, it can be seen that a significant part of the receptor protrudes from the membrane to the exterior. The ‘bottleneck’ or gate is located at the level of the lipid bilayer. An additional portion of the receptor sits on the cytosolic surface of the receptor and may be important for its ion selectivity.

² Because protein crystals could only be grown after their removal by limited proteolysis or recombinant DNA techniques. Example: The structure of the voltage-gated K^+ channel we have seen before.

³ Which, like muscle cells, are derived from the mesoderm during embryonic development.

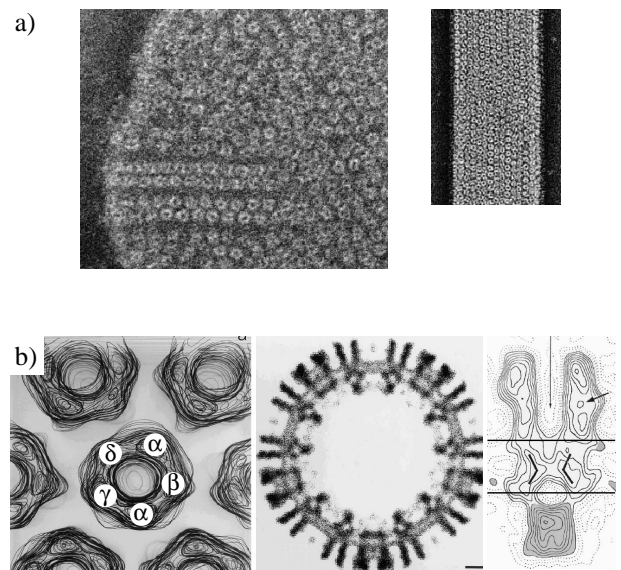


Figure 9.3. Electron microscopy and ‘electron crystallography’ of the nicotinic acetylcholine receptor (NAR). a: NAR channels in liposome membranes. On the left, they are mostly randomly oriented (but some form a more regular pattern). On the right, a regularly packed ‘two-dimensional crystal’ has formed. Such samples can be used to obtain a three-dimensional structure at low resolution by electron crystallography. b: Electron crystallographic structure, represented as density contour maps. Left: Top view. Middle, right: Side view. The bilayer and the portions of the receptor protruding from it into both directions are visible. The arrow in the right frame points to the acetylcholine binding site.

The bottleneck is lined by 5 α -helices (one each from the 5 subunits). In the closed state, they touch upon each other, making direct hydrophobic contacts via some leucine residues (Figure 9.4). These helices move apart by a considerable distance during channel opening, creating a rather large free lumen.

9.1.2. Location of the acetylcholine binding site

The acetylcholine binding site was mapped onto the structure by comparing the electron densities of ligand-bound and unbound samples. This site has also been extensively characterized with biochemical methods, which allow the assignment of amino acid residues involved in ligand binding. One important technique consists in affinity labelling. Figure 9.5 summarizes one such study.

The compound used is a derivative of acetylcholine. It contains tritium (3H) and thus is radioactive; it also contains a photo-activatable reactive group (blue). If allowed to bind to the receptor and illuminated with UV light, this group will attach itself onto anything in the vicinity, including amino acid residues, even ones of very low intrinsic reactivity. This will lead to the incorporation of the radioactive label into the receptor. The gel shows the labelled α and γ

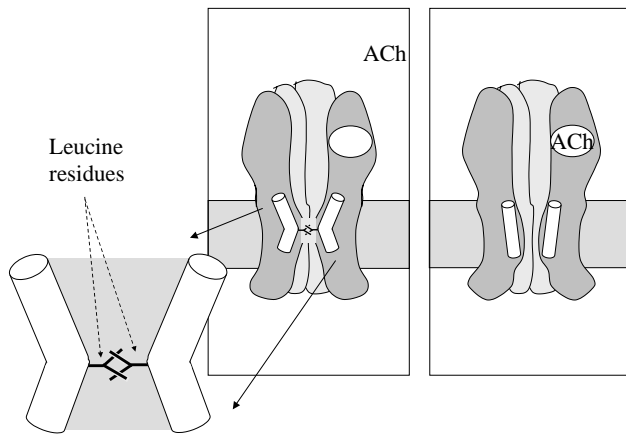


Figure 9.4. Closed and open states of the nicotinic acetylcholine receptor. The muscle type NAR has two binding sites for acetylcholine (ACh); the approximate location of one site is indicated. The gate is located at the level of the bilayer; in the closed state, it is held together by the interaction of leucine residues on the helices that surround the channel lumen.

subunits (as well as a third band that reportedly is a degradation product of the γ chain). Importantly, this labeling occurs selectively at the agonist binding site, since it is largely suppressed in the presence of excess carbachol, which is a synthetic agonist closely related to acetylcholine (see later).

After labelling, the chains were separated⁴ and individually cleaved with a site-specifically acting protease (V8)⁵. Figure 9.5b shows that, in the α chain, the bulk of the radioactivity is associated with one of the proteolytic fragments (named α V8-20). However, specific labelling (i.e., labelling that can be suppressed by the specific competitor carbamoylcholine) also occurs in other fragments.

Individual proteolytic fragments, in turn, were isolated by electrophoresis and further purified by HPLC, and the labelled residues within them were identified by protein sequencing (Figure 9.5c). With the fragment shown, most of the label is found attached to two adjacent residues (Leu109 and Val 110).

While with the reagent used here only the α and the γ chains were strongly labelled, previous experiments (using different affinity probes with reactive groups attached to different sites of the acetylcholine molecule) have provided evidence that the δ chain is involved in ligand binding as well. In fact, there are two binding sites for acetylcholine in muscle-type NAR, located at the interfaces of the two α chains with the γ and the δ chains, respectively (cf. Figure 9.3b).

⁴By SDS-PAGE. Boiling the samples in SDS will dissociate the subunits, since they are not covalently linked.

⁵V8 (isolated from the bacterium *Staphylococcus aureus*) cleaves after aspartate and glutamate residues.

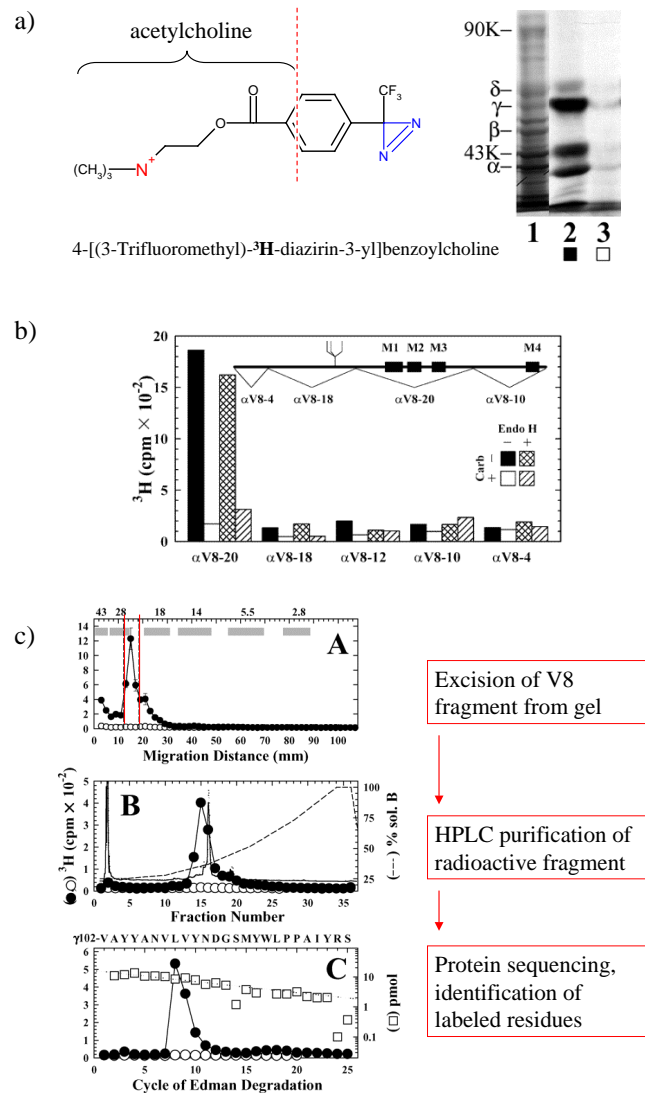


Figure 9.5. Mapping of the acetylcholine binding site by affinity labeling. a: Structure of the photoactivatable ligand used. The activatable group is indicated in blue. Right: SDS-PAGE. Lane 1: Unlabeled control; lane 2: Labeled sample; lane 3: Same as lane 2 but also containing unlabelled carbamoylcholine in excess. b: Incorporation of label into proteolytic fragments of the α chain. Black bars: Labeled in the absence of carbamoylcholine, white bars: Labeled with an excess of carbamoylcholine present. c: Identification of individual labelled amino acid residues by Edman degradation. See text for further details.

9.1.3. The nature of the receptor-ligand interaction

Virtually all agonists and antagonists at the NAR share the positive charge of acetylcholine, most commonly (as in acetylcholine) in the form of a quaternary amino group⁶. What role does this positive charge have? The agonist binding site of NAR is not rich in negative charges (aspartate or glutamate residues) but instead has multiple aromatic

⁶Sometimes a tertiary amine, though; cf. mecamylamine, which was discussed earlier in the chapter on pharmacokinetics.

side chains. These can bind to cations by a peculiar mechanism, called the ‘cation- π ’ interaction, in which the fixed charge of the cation is accommodated by the mobile π electrons of the aromatic ring. Using a quite sophisticated set of methods, it was determined that this mechanism indeed is responsible for the binding of acetylcholine to the NAR. These experiments were carried out as follows (Figure 9.6):

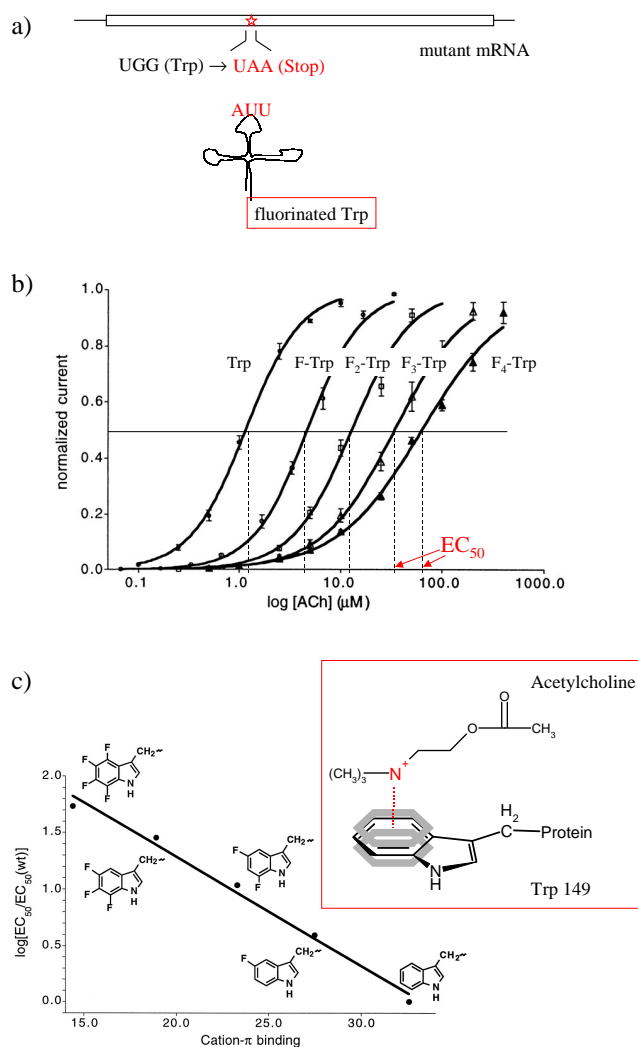


Figure 9.6. Cation- π interaction in the binding of acetylcholine to the nicotinic acetylcholine receptor. a: Incorporation of fluorinated tryptophan residues into NAR α chains *in vivo*. Individual tryptophan codons were replaced by amber stop codons (UAA). The mRNA was obtained by transcription *in vitro* and injected into *Xenopus laevis* (frog) oocytes, along with a suppressor tRNA that had been acylated with the synthetic fluorinated trp. b: Oocytes expressing the modified NAR were analyzed by patch clamp. Currents are plotted as functions of acetylcholine concentration. The EC_{50} increases monotonously with the extent of fluorination. c: Replot of the EC_{50} as a function of a derived parameter that describes the strength of the cation- π interaction (the derivation is beyond me).

1. The codons of individual tryptophan residues in the cloned α chain were exchanged for an amber stop codon (TAA) and the mutant mRNA was obtained by *in vitro* transcription.
2. A suppressor tRNA – a tRNA carrying a mutant anticodon that recognizes a complementary stop codon – was likewise generated *in vitro* and synthetically acylated with various fluorinated derivatives of tryptophan. This tRNA will selectively incorporate its amino acyl cargo at the mutant stop codon.
3. The mRNA and the tRNA were both injected into frog oocytes.
4. The frog oocytes expressing the mutant channels were studied using the ‘patch-clamp’ method, which allows the characterization of single channels on intact cells⁷.

The non-natural tryptophan derivatives incorporated at the mutant stop codon contained various numbers of fluorine as substituents at the benzene ring that is part of the indole in the tryptophan side chain (cf. Figure 9.6c). In Figure 9.6b, it is shown that with increasing numbers of fluorine substituents the sensitivity of the receptor to activation by acetylcholine decreased continuously, as ever higher amounts of the agonist were required to achieve half-maximal response.

Fluorine is very small and is considered not to cause major steric changes when substituted for hydrogen. However, it is very strongly electronegative and will therefore pull π electrons out of the ring; this will weaken the cation- π interaction. Figure 9.6c plots the observed receptor sensitivities (as logarithms of the EC_{50} ⁸) against a theoretical parameter that describes the intensity of the cation- π interaction for tryptophan and its fluorinated derivatives. The correlation suggests that this interaction indeed is very important for the binding of acetylcholine to the NAR. By comparing the effects of substituting different tryptophan residues, it was also determined that the most significant single residue in this interaction is tryptophan 149 in the α chain.

9.1.4. Receptor desensitization

An aspect of NAR function that is very important in its pharmacological manipulation is receptor desensitization. This phenomenon can be experimentally demonstrated in the set-up depicted in Figure 9.7: Stimulating electrodes produce presynaptic action potentials, which induce release of acetylcholine into the synaptic cleft. Acetylcholine will trigger postsynaptic action potentials, which in turn are de-

⁷Or on excised ‘patches’ of membrane, in which case the conditions on both sides of the membrane can be controlled.

⁸ EC_{50} : Agonist concentration required for 50% of maximal effect (see the pharmacodynamics chapter).

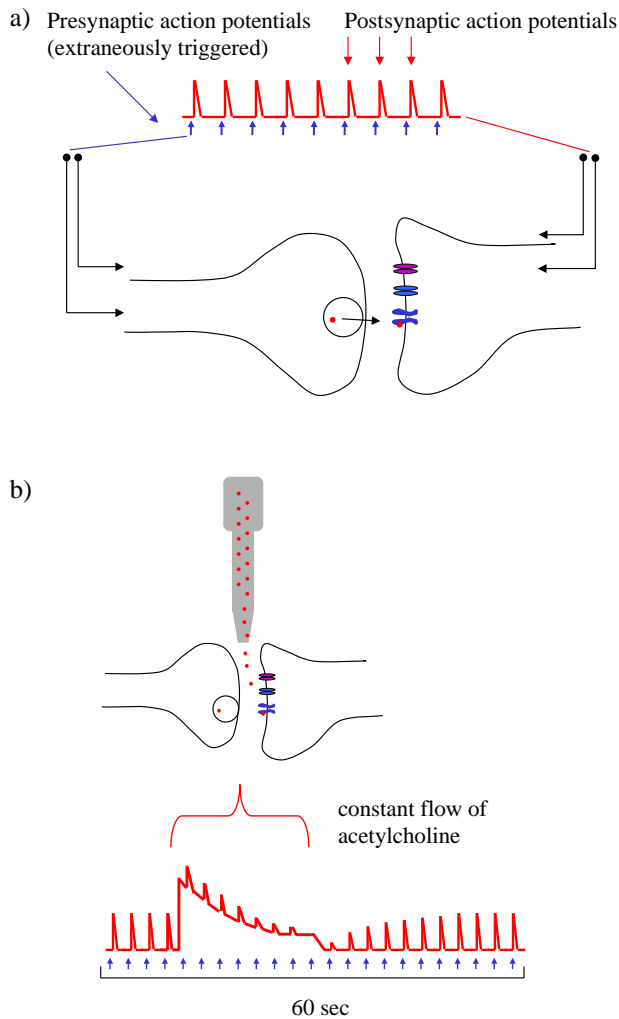


Figure 9.7. Desensitization of the nicotinic acetylcholine receptor. a: Experimental setup. Presynaptic action potentials were triggered, and postsynaptic ones were detected using two separate sets of electrodes. b: Perfusion of the synapse with extraneous acetylcholine (during the time interval indicated by the horizontal brace) brings about a sudden depolarization. Continuous presence of acetylcholine rapidly suppresses the response to presynaptic action potentials; also, the membrane potential returns to levels near the resting potential. Discontinuation of the acetylcholine results in restoration of response to presynaptic stimuli.

tected with a second pair of electrodes. These postsynaptic action potentials attain a reproducible, fairly constant height, and they are of very short duration, which reflects swift removal of acetylcholine by cholinesterase. If extraneous acetylcholine is applied to this system (in a continuous fashion, so as to keep up a constant level in spite of cholinesterase activity), there is a steep initial depolarization of the membrane, which then peters out over a time interval of a few seconds. Additional presynaptic stimuli during this time period are ineffectual. If the extrinsic supply of acetylcholine is discontinued, cholinesterase rapidly cleans up, the membrane completely repolarizes, and its

sensitivity to presynaptic triggers is restored in another few seconds.

Receptor desensitization can be understood in terms of a model of channel function that is similar to what we have seen before with voltage-gated channels (Figure 9.8). The receptor can cycle between three functional states: Resting (no current), open (current), and inactivated (no current). In the absence of ligand, the resting state is the most stable one, so most receptors will be in this state. Ligand binding favours both the open and the inactivated states over the resting state. Therefore, at sufficiently high ligand concentration, essentially all receptor molecules will leave the resting state. Importantly, the open state is kinetically favoured over the inactivated state, which is a fancy way of saying that opening is faster than inactivation. Thus, most channels will initially enter the open state (although some will become directly inactivated). However, the inactivated state is thermodynamically favoured over the open state, which means it is more stable than the latter. In fact, at high concentrations of ligand, it is the most stable of all three states, and therefore upon continuous exposure to acetylcholine all the receptors that initially opened eventually wind up in the inactivated state. This explains the above observation of membrane repolarization and insensitivity during perfusion of the synapse with acetylcholine.

9.2. Cholinergic agonists

Drugs that stimulate acetylcholine receptors are conventionally called ‘direct agonists’, as opposed to ‘indirect agonists’, which are inhibitors of acetylcholinesterase (see below). Direct cholinergic agonists are used in a variety of clinical applications. Acetylcholine itself is not a very use-

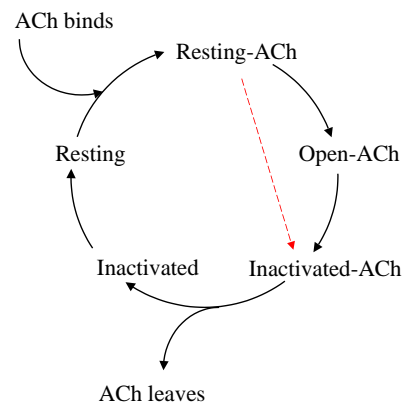


Figure 9.8. Three-state model of nicotinic acetylcholine receptor action. The resting state is the most stable one in the absence of ligand. Ligand binding promotes transition first to the open state and from there to the inactivated state (some molecules may actually inactivate directly, red arrow). Reactivation requires dissociation of acetylcholine, which is promoted by the scavenging of acetylcholine by cholinesterase.

ful drug because it gets so rapidly hydrolysed. Just like in the experiment above (Figure 9.7), its action *in vivo* subsides as a matter of seconds after discontinuation. Most cholinergic agonists that are in clinical use are partially or completely resistant to degradation by cholinesterase and thus will remain active for extended periods of time.

9.2.1. Muscarinic agonists

Two such agonists are shown in Figure 9.9. In the structure of carbamoylcholine, the acetyl group is replaced by a carbamoyl group. This agonist is only very slowly degraded by cholinesterase. It resembles acetylcholine in being active at both muscarinic and nicotinic synapses. However, the muscarinic effects are stronger, and carbamoylcholine is being used clinically to stimulate intestinal and urinary bladder motility in transient states of paralysis that may occur following surgical procedures. Metacholine retains the acetyl group; its lower susceptibility to cholinesterase is due to a methyl group that sterically hinders the enzyme. The methyl group, at the same time, renders it selectively active on muscarinic synapses. It is used for the same purposes as carbamoylcholine.

Both carbamoylcholine and metacholine are esters and as such highly susceptible to less specific esterases in the intestine and liver, so that they cannot be applied orally. Other cholinergic agonists, however, do not possess an ester bond at all, and many of those are indeed active after oral application.

Figure 9.10 shows two agonists that act selectively on muscarinic synapses: Muscarine (surprise!) and pilocarpine. Both of these compounds have rather remote similarity with each other or with acetylcholine. Muscarine does not have an ester bond and is active orally. It is, however, not used therapeutically – rather, it is a poison found in toadstool⁹ and other mushrooms. It will produce the effects – bronchial constriction, hypersalivation, intestinal cramps –

| | N | M | CE |
|--|-----|-----|-----|
| $\text{H}_3\text{C}-\text{C}(=\text{O})-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ | +++ | +++ | +++ |
| Acetylcholine | | | |
| $\text{H}_2\text{N}-\text{C}(=\text{O})-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ | ++ | +++ | (-) |
| Carbamoylcholine | | | |
| $\text{H}_3\text{C}-\text{C}(=\text{O})-\text{O}-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ | - | +++ | + |
| Metacholine | | | |

Figure 9.9. Structures of the cholinergic agonists carbamoylcholine and metacholine, their relative effects on nicotinic (N) and muscarinic (M) synapses, and their susceptibilities to cholinesterase (CE).

that can be predicted from the distribution and functional effects of the muscarinic receptors that were discussed in the preceding chapter.

Pilocarpine, in contrast to muscarine, is being used therapeutically – mostly for local application to the eye. It will cause both miosis (by action on the sphincter or constrictor muscle of the iris) and accommodation of the eye lens for seeing close, by acting on another (ciliary) muscle. These muscle movements will decongest a tiny canal which is located right behind the ciliary muscle¹⁰ and thereby facilitate the efflux of fluid from the eye. Pilocarpine is thus used in glaucoma, a disease that is caused by pathologically increased pressure within the eye.

Pilocarpine is also used orally – not in clinical medicine, but by some natives of South America, who for some reason appear to appreciate the salivation it induces and chew the leaves of the shrub that contain it. It does not seem to cause major toxic effects by ingestion – possibly it is again unstable in the intestine because of its internal ester (lactone) bond. However, I assume that it can be taken up to some extent across the mucous membranes.

9.2.2. Nicotinic agonists

Nicotine and lobeline (Figure 9.11) are as well found in plants. Both act as agonists on nicotinic synapses, and they share the feature of being fairly hydrophobic. The amino groups in both of them are tertiary rather than quaternary, so that both are capable of non-ionic diffusion, enabling them to pass across membrane barriers. With nicotine, this is apparent in its effect on the central nervous system (along with that upon the peripheral autonomic ganglia). In addition, nicotine is also used in plasters and chewing gums¹¹ by those who want to quit smoking, which is evidence that it

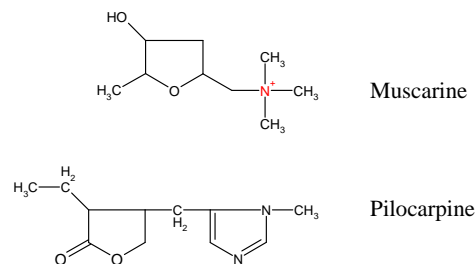


Figure 9.10. Structures of the muscarinic agonists muscarine and pilocarpine.

⁹Muscarine, however, is *not* the major poison of toadstool; that spot is taken by muscimol, which has *antimuscarinic* effect! Treatment of *Amanita muscaria* intoxication therefore will need to augment, not decrease muscarinic action.

¹⁰The canal of Schlemm – at least that is its name in German literature.

¹¹In former times, chewing or sniffing tobacco were quite popular, further illustrating that nicotine can be taken up across membrane barriers.

can cross mucous membranes and even the skin with ease. In contrast to nicotine, dimethylpiperazinium has a quaternary amino group. It is therefore permanently ionic and does not easily cross the blood-brain barrier. It does not have any clinical applications I'm aware of, but it is used in research with experimental animals for selective stimulation of the autonomic ganglia in the absence of simultaneous central effects.

The peripheral autonomic effects of nicotine and similar agents are somewhat irregular, due to the fact that both sympathetic and parasympathetic ganglia are stimulated, which will result in partially antagonistic actions. However, in general, the effects on the intestine will be mainly parasympathetic, which can be nicely observed in small boys smoking their first cigars. Conversely, the effect on the cardiovascular system is predominantly sympathetic, which may be related to the fact that not only the ganglia but also the adrenal glands are being stimulated. With the dosages that are required for the autonomic effects, the functional changes at the motor endplate (= neuromuscular synapse) are irrelevant; at very high concentrations, depolarizing blockade (see later) may be induced.

9.3. Cholinergic antagonists

Cholinergic antagonists have a larger therapeutic role than agonists. Again, we can distinguish drugs that selectively affect nicotinic and muscarinic receptors.

9.3.1. Muscarinic antagonists

The classical muscarinic antagonists are atropine and the closely similar scopolamine. Atropine (Figure 9.12) will to some extent enter the brain and cause upheaval there as well – this is reflected in the German name of the plant containing it (*Tollkirsche* = "crazy-cherry"). It is used for local application to the eye (widening the pupil, relaxing the ciliary muscle in diagnostic procedures), and during surgery to relax the airways and suppress salivation, both of which will

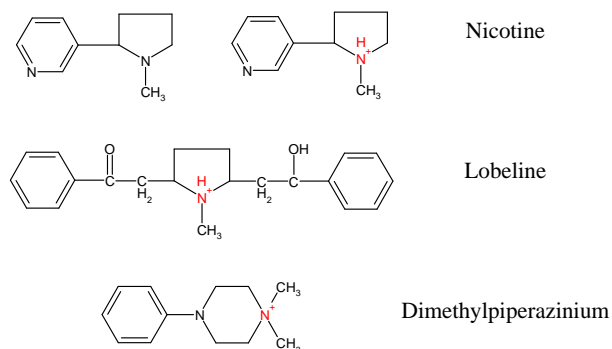


Figure 9.11. Structures of the nicotinic agonists nicotine (deprotonated and protonated), lobeline, and dimethylpiperazinium.

help to avoid respiratory problems during narcosis. Ipratropium is used for oral and inhalation treatment to relax the airways in asthma, and occasionally to speed up a slow atrioventricular node in the heart (cf. the preceding chapter). It is preferred over atropine in most applications because it does not quite as easily cross the blood brain barrier – by now, you should be able to tell why when looking at the structure.

Benztropine is the most hydrophobic one of the three drugs in Figure 9.12 and accordingly distributes into the brain most easily. It is used as a component in the treatment of Parkinson's disease; anticholinergic drugs augment¹² the action of dopaminergic drugs (such as L-DOPA) that remain the mainstay of therapy in this disease.

9.3.2. Nicotinic antagonists

Due to the molecular differences between NAR at the neuromuscular junction and the autonomic ganglia, many nicotinic antagonists are quite selective for one over the other. Antagonists that are specific for the ganglia – 'ganglion blockers' – were among the first drugs to be used effectively for the treatment of hypertonia. However, they have now been nearly entirely abandoned, which is due to their rather numerous side effects which result from the blockade of both the sympathetic and the parasympathetic systems. We have seen the structures of hexamethonium and mecamylamine before in the chapter on pharmacokinetics – you will note the similarity between their pharmacokinetic characteristics and those described in this chapter for the other cholinergic agonists and antagonists. Another interesting structure is that of trimethaphan (Figure 9.13), which

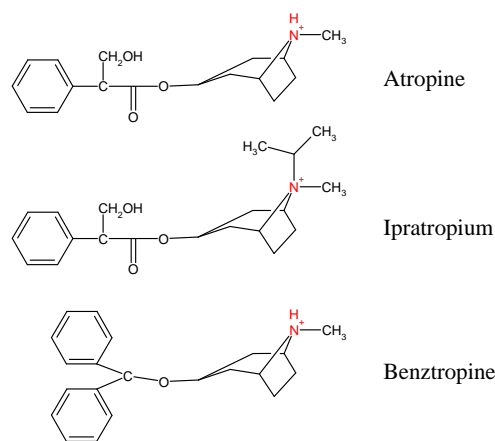


Figure 9.12. Structures of the muscarinic antagonists atropine, ipratropium, and benztropine.

¹²This effect is due to the antagonistic arrangement of dopaminergic and cholinergic neurons in certain areas of the brain. In addition, benztropine also seems to inhibit the presynaptic reuptake of dopamine (see the subsequent chapter).

is still sometimes used in a condition called hypertensive crisis. In this molecule, the place of the quaternary amino group is taken by a sulfonium ion, which consists of a sulfur atom bound to three aliphatic substituents.

Nicotinic antagonists with specificity for the neuromuscular junction will cause paralysis. These agents are discussed in the following section.

9.3.3. Muscle relaxants

Muscle relaxants suppress muscle contraction in response to stimulation by the motoneurons. They may be either antagonists or agonists of nicotinic acetylcholine receptors. Muscle relaxants are routinely used in anesthesiology. To understand this application, consider that narcosis serves two distinct purposes:

1. Suppression of pain perception,
2. Suppression of muscle motility – after all, we don't want the patient to jump of the table and sleep walk around the operation theater.

For the first purpose, it is sufficient to abolish the activity of the higher centres in the brain, whereas the complete abolishment of reflex motility requires suppression of more or less the entire central nervous system. Although the latter can be achieved with narcotic gases (such as diethylether and its more modern congeners) alone, it requires higher dosages than are required for pain suppression only. The use of muscle relaxants makes it possible to reduce the amount of narcotic gases needed, and therefore to limit the associated risks and side effects.

9.3.4. Nicotinic antagonists used as muscle relaxants

The first muscle relaxant to be discovered and clinically used was d-tubocurarine (Figure 9.14a), which is found in curare, an arrow poison that was used by South American natives. Quite a bit of imagination is required to spot any structural resemblance to acetylcholine; d-tubocurarine is much larger and contains not one but two quaternary amines within a rather large, rigid ring structure. The resemblance is more readily spotted with the more recent, synthetic compound pancuronium (Figure 9.14a). This molecule has two acetylcholine moieties, embedded in a

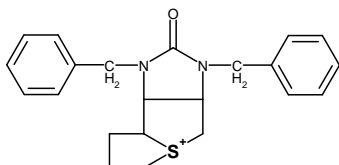


Figure 9.13. Structure of the ganglion-blocking agent trimetaphan.

larger structure that again looks rigid but otherwise does not have much similarity to d-tubocurarine.

Both pancuronium and d-tubocurarine act by binding to the NAR in a way competitive with acetylcholine. In both cases, the presence of both positive charges is important for activity. Yet, it is questionable whether both cationic groups bind simultaneously to the two acetylcholine binding sites found (at the two α chains) of the NAR, since the distance between those should substantially exceed the one between the two charges in the antagonist molecules.

9.3.5. Depolarizing muscle relaxants

While both d-tubocurarine and pancuronium prevent opening of the NAR, another class of muscle relaxants effects neuromuscular blockade by *triggering* the opening of NAR and, accordingly, membrane depolarization. Thus, these drugs are not antagonists but agonists. In fact, this type of blockade – called ‘depolarizing block’ – can be achieved with acetylcholine itself, if used at dosages exceeding those that occur physiologically¹³. The only agent in widespread use is succinyl-bis-choline (also called succinylcholine);

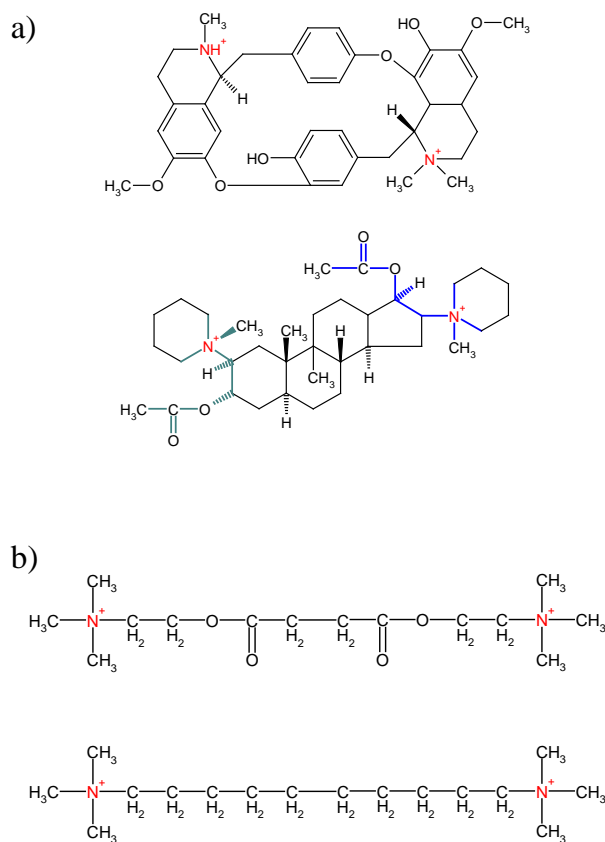


Figure 9.14. Structures of muscle-relaxants. a: d-Tubocurarine (top) and pancuronium. The two acetylcholine moieties in the pancuronium molecule are highlighted. b: Structures of the ‘depolarizing muscle relaxants’ succinylcholine (top) and decamethonium (bottom).

this is just two acetylcholine molecules joined end on (Figure 9.14b). Another agonist is decamethonium, which is a longer analog of hexamethonium; however, the latter is an antagonist, not an agonist. One aspect of this blockade consists in the desensitization of the NAR – continuous presence of acetylcholine or another agonist will lead to accumulation of the receptor in the inactivated state (cf. Figure 9.7, above). However, other mechanisms must be involved, too, which follows from the following observations:

1. The membrane stays partially depolarized during the presence of the agonist. If all receptors were fixed in the inactivated state, the membrane should be completely repolarized.
2. If muscle cells have been cut off from their neural input for a couple of weeks, they will express larger numbers of NAR molecules, even outside those membrane regions that were previously in contact with the nerve cells. If acetylcholine or another depolarizing agent is applied to these cells, they will respond with maximum contraction, and no blockade will ensue. Thus, some regulatory mechanism that suppresses muscle contraction upon sustained membrane depolarization in normal cells seems to be lost concomitantly with the denervation.

Succinylcholine has a more sustained action at the motor endplate than acetylcholine has because it is insensitive to the acetylcholinesterase found in the synapse. There is, however, a second variety of cholinesterase that circulates in the blood plasma, also referred to as butyrylcholinesterase¹⁴, which cleaves succinylcholine within minutes. This moderately rapid inactivation makes it possible to control the degree of muscle relaxation by adjusting the infusion rate of the agent; after discontinuation, the remaining succinylcholine will be swiftly hydrolysed, and the block will subside¹⁵.

¹³ Maximal release of acetylcholine by the motoneurons occurs in tetanus, due to the toxin-mediated inactivation of glycinergic neurons that normally inhibit them. This results not in blockade but maximal muscle activity, so strong that bone fractures are commonly observed. However, blockade can be observed with inhibitors of acetylcholine esterase (see below).

¹⁴ This name reflects that the activity of this enzyme used to be assayed with butyrylcholine (I'm not sure if that model substrate is still in use). However, the enzyme also cleaves acetylcholine and many other natural or synthetic choline esters, just as β -galactosidase cleaves both lactose and many synthetic β -galactosides, including the blue X-Gal that you may have seen in the lab.

¹⁵ Note that both cleavage products – succinate and choline – are physiological metabolites. Succinylcholine will leave no traces – a great way to dispose of intrusive mothers-in-law. See? This class is *useful*!

9.4. Cholinesterase antagonists

The last group of agents that affect cholinergic synaptic transmission are blockers of cholinesterase. This enzyme has a catalytic mechanism that is analogous to that of chymotrypsin and related proteases. In chymotrypsin, there is a 'catalytic triad', consisting of a serine, a histidine, and an aspartic acid side chain in the active site. The only difference with cholinesterase is that a glutamate residue replaces the aspartate – and this difference is insignificant, because glutamate and aspartate share a carboxyl group, which is the essential feature for catalysis.

Within this catalytic triad, the glutamate and the histidine residues cooperate to effect deprotonation of the hydroxyl group in the serine¹⁶ side chain (Figure 9.15). The anionic oxygen is a powerful nucleophile that will readily attack the

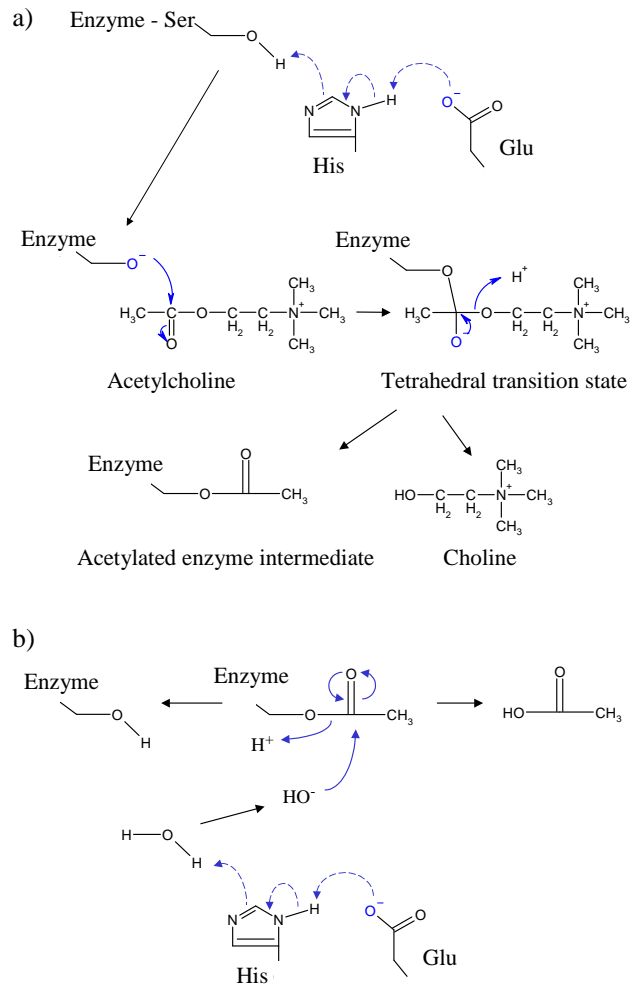


Figure 9.15. Catalytic mechanism of acetylcholinesterase. a: A catalytic triad yields a deprotonated serine, which attacks acetylcholine and winds up as an acetylated intermediate. Transfer of the acetyl group to the enzyme involves a tetrahedral transition state. b: Hydrolysis of the acetyl group is facilitated by an analogous mechanism, which involves a hydroxide anion as the nucleophile.

carbonyl carbon of the ester bond in acetylcholine. This will release choline and leave the acetyl group attached (via another ester bond) to the enzyme. In order to reactivate the enzyme, the ester bond must be cleaved again. The nucleophile that is utilized in this step is a plain hydroxide anion, generated by the deprotonation of water according to the same mechanism as seen before for the serine.

9.4.1. Chemical groups of cholinesterase inhibitors

The two-step mechanism of acetylcholinesterase provides the basis for the mode of action of most cholinesterase inhibitors. This is shown for the inhibitor neostigmine in Figure 9.16. Initial cleavage of the inhibitor works just fine; however, the carbamoylated intermediate is far more stable than the acetylated one and is only very sluggishly hydrolysed to regenerate the active enzyme. In fact, the same reaction as with neostigmine occurs with carbamoylcholine, too. Thus, carbamoylcholine is both an agonist at the receptor and an inhibitor of acetylcholinesterase. After stoichiometric reaction of the enzyme with the substrate, any further reaction will be very slow, and since the drug will be present in large excess over the enzyme, carbamoylcholine will appear resistant to cholinesterase.

A different class of acetylcholinesterase inhibitors are the so-called organophosphates (Figure 9.17). While acetylcholine and the carbamoyl-based esterase inhibitors have a trihedral structure at the site of attack (the carbonyl group) and assume a tetrahedral structure only in the transition state of the reaction, the organo-phosphates have a tetrahedral structure right from start. Since enzymes commonly have a high binding affinity for the transition state, this ex-

plains that organophosphate inhibitors bind very avidly to acetylcholinesterase. They are, accordingly, extraordinarily toxic, and their small molecular size and hydrophobic character enable them to be taken up quickly by inhalation or even across the skin.

For one of the organophosphates, diisopropylfluorophosphate (DFP), the reaction mechanism is outlined in Figure 9.17a. Fluorine makes an excellent leaving group and is substituted by the active site serine. It is also found in the ‘nerve gas’ soman. A different leaving group (cyanide) is found in the nerve gas tabun (Figure 9.17b).

Another factor that contributes to the high level of toxicity of the organophosphates is the stability of the phosphate ester bond formed with the enzyme: The enzyme does not spontaneously hydrolyse itself, not even slowly as is the case with the carbamates. Reactivation can be accomplished with an extraneous nucleophile such as hydroxylamine (Figure 9.18). While the latter works fine in vitro, it is too toxic for use in vivo. However, analogs such as prali-

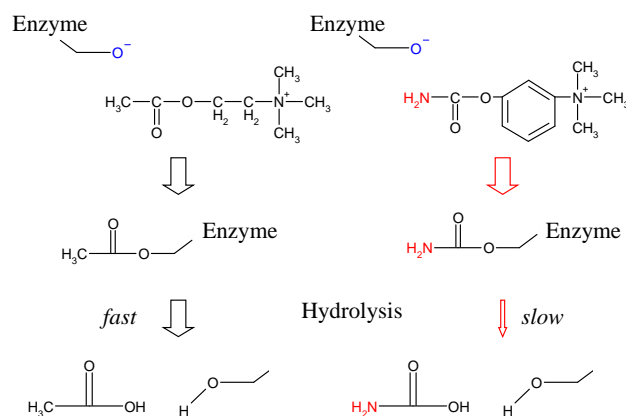


Figure 9.16. Inhibition of acetylcholinesterase by neostigmine (top, right). The first step – carbamoylation of the enzyme – occurs readily, but the subsequent hydrolysis is very slow. Acetylcholine is shown on the left for comparison.

¹⁶In some proteases, e.g. proteasomes, serine is replaced by a threonine. Again, this change is basically irrelevant, as both serine and threonine share the hydroxyl group.

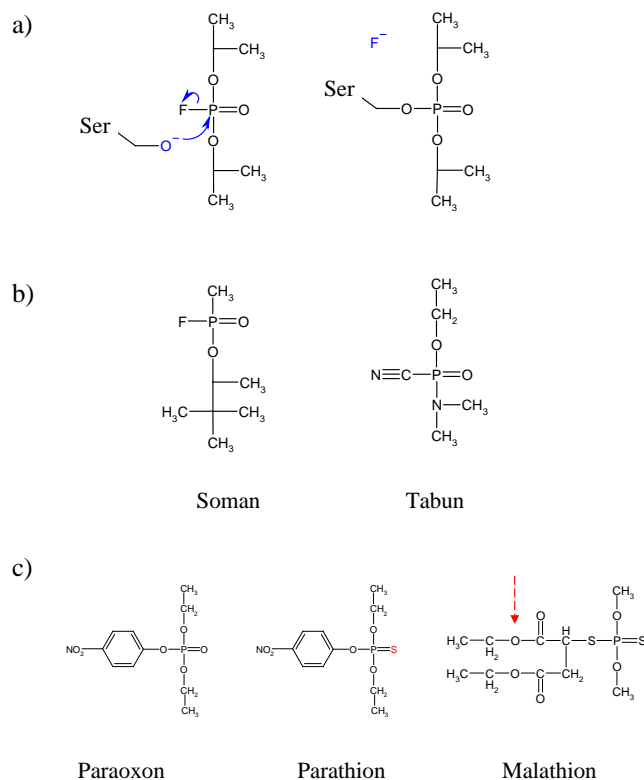


Figure 9.17. Organophosphate inhibitors of acetylcholinesterase. a: The catalytic mechanism, shown here for diisopropylfluorophosphate (DFP). b: Structures of soman and tabun. Like DFP, these were developed during world war II as ‘nerve gases’. c: Structures of the insecticides parathion and malathion, and of paraoxon, which is the active metabolite of parathion. (Malathion likewise requires conversion to malaaxon.) The arrow above the malathion structure indicates the esterase cleavage sites in its leaving group; esterase cleavage occurs in human plasma and renders the molecule non-toxic.

doxime and obidoxime have been developed that steer the hydroxylamine reactive group to the active site of acetylcholinesterase. This means they are effective at far lower concentrations and can indeed be used in vivo for the treatment of organophosphate poisoning¹⁷.

The cholinesterase inhibitors have multiple practical applications – from medicine to warfare. Physostigmine was actually the first practically used cholinesterase antagonist. To describe its initial use as ‘medical’, however, would mean a bit of a stretch – instead, the seed¹⁸ containing it was used to extort confessions from persons accused of crimes or witchcraft in Guinea. This seed used to be called the ‘ordeal bean’ by the local people.

9.4.2. Applications of cholinesterase inhibitors

Physostigmine and other carbamate derivatives such as neostigmine, and edrophonium (which is a non-covalent antagonist; Figure 9.19) are preferred in medicine over organophosphates because of their more controllable (and self-terminating) activity. One important application is in a disease called ‘Myasthenia gravis pseudoparalytica’, which means ‘severe, quasi-paralytic muscle weakness’. This disease is due to the pathological formation of neutralizing (i.e., inactivating) antibodies against the NAR of the muscle cells¹⁹. The post-synaptic membranes will then be-

come less sensitive to the acetylcholine stimulus. Prolongation of the lifetime of acetylcholine by partial blockade of cholinesterase is used (with varying success) to compensate for this. Another application is in glaucoma, as described above for direct cholinergic agonists.

Cholinesterase inhibitors of both the carbamate and the organophosphate type are very important as insecticides. Such agents include parathion (which is first converted metabolically to paraoxon; Figure 9.17c), malathion, and carbaryl. Parathion is very poisonous for both insects and vertebrates (including *homo sapiens*), and therefore has largely been abandoned. Carbaryl binds more avidly to insect acetylcholinesterases and therefore has better selectivity. Malathion achieves better selectivity by the inclusion of ester bonds within its peculiar (thiomalatediethyl ester) leaving group. Cleavage of those (by esterases other than cholinesterase) inactivates it and appears to proceed much more rapidly in mammals than in insects.

Organophosphates are active not only against acetylcholinesterase but also serine proteases – which is obviously due to the shared catalytic mechanism. DFP is actually being used as a protease inhibitor in biotechnology. Another inhibitor that shares its mode of action but is less dangerous (because it is not volatile, and the enzyme adducts it forms are less stable) is PMSF (phenylmethylsulfonyl fluoride). You may have encountered it in one or the other research lab; it is commonly added to crude cell extracts in order to minimize enzymatic breakdown of proteins during purification.

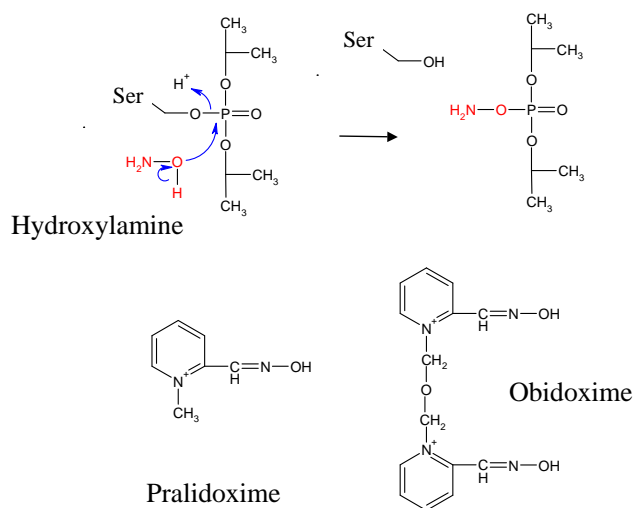


Figure 9.18. Reactivation of DFP-reacted cholinesterase with hydroxylamine, and structures of the two related drugs pralidoxime and obidoxime, which are clinically used in organophosphate poisoning.

¹⁷ This treatment must be combined with more immediately effective measures such as mechanic respiration. The most serious and life-threatening consequence of general neuromuscular blockade is, of course, the inability to breathe.

¹⁸ The plant is called *physostigma venenosum* (venenum = lat. poison)

¹⁹ A similar disease, called *Lambert Eaton syndrome*, is caused by anti-

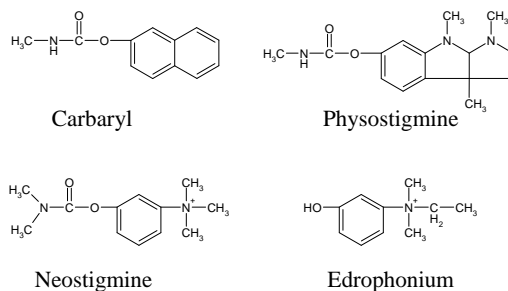


Figure 9.19. Structures of three carbamate type cholinesterase blockers, and of the non-covalent blocker edrophonium.

bodies against the presynaptic calcium channel that triggers the release of acetylcholine.

(Notes)

Chapter 10. Pharmacology of catecholamines and of serotonin

The catecholamines (named for the catechol moiety that is part of their structure, Figure 10.1) are important in both the peripheral autonomic system and the central nervous system. Key functions in the periphery are regulation of heart rate and blood pressure. In the brain, they are involved in the regulation of posture and movement, and of psychical functions such as mood and alertness.

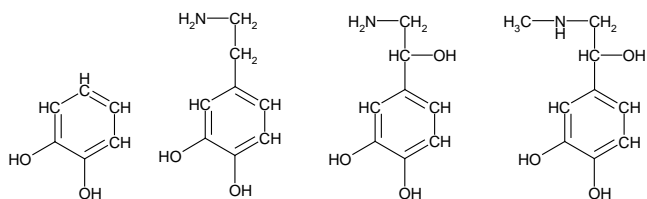


Figure 10.1. Structures of catechol, dopamine, norepinephrine, and epinephrine (from left to right).

Although all three mediators occur both peripherally and centrally, dopamine and norepinephrine are the main ones found as transmitters in the brain, whereas norepinephrine and epinephrine are more important than dopamine in the periphery. Norepinephrine occurs in both synapses and in the adrenal gland, whereas epinephrine is mainly found in the adrenal gland and thus really is a hormone more than a transmitter.

Serotonin is a mediator similar to the catecholamines that is derived from tryptophan rather than tyrosine (Figure 10.2b). It also occurs as a mediator in the periphery, but its major interest in clinical pharmacology is due to its role in the central nervous system, where it shares with the catecholamines in the regulation of mood. The functional organization of synapses for both catecholamines and serotonin is closely similar, so that it is useful to discuss them side-by-side.

10.1. Biosynthesis and degradation of catecholamines

The catecholamines – dopamine, norepinephrine, and epinephrine are successively derived from tyrosine. Synthesis occurs in the nerve terminals and in the adrenal gland. Tyrosine hydroxylase catalyzes the first step (Figure 10.2a) and is the major site of regulation (inhibition by dopamine and noradrenaline, activation by cAMP). This step gives rise to 3,4-dihydroxyphenylalanine (L-DOPA), which in turn is a substrate for L-aromatic acid decarboxylase. De-

carboxylation yields the first mediator, dopamine. The substrate specificity of the decarboxylase is rather low, and it thus will also accept 5-hydroxytryptophan (the precursor of serotonin, Figure 10.2a) and a variety of synthetic analogs, as we shall see later. Further hydroxylation of dopamine leads to norepinephrine, and methylation to epinephrine.

The biosynthesis of serotonin is similar to that of dopamine and also involves enzymatic hydroxylation and subsequent decarboxylation (Figure 10.2b).

Catecholamines and serotonin also share the major mode of degradation. Both are substrates for monoamine oxidase (MAO), although one subtype of this enzyme (MAO A) has a preference for catecholamines, whereas the other (MAO

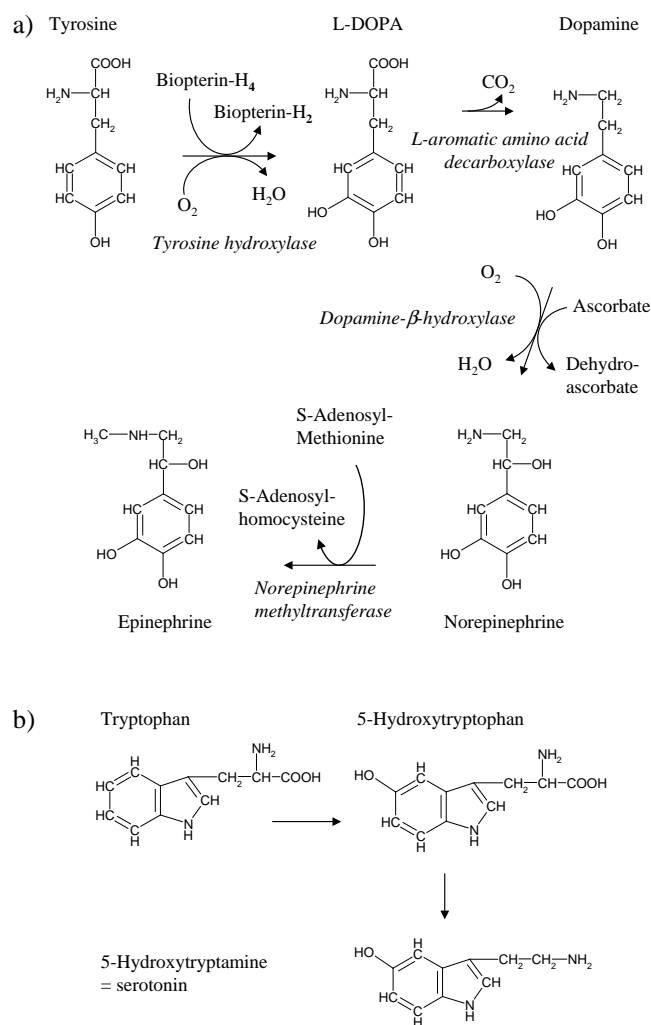


Figure 10.2. Biosynthesis of catecholamines (a) and of serotonin (b).

B) acts more efficiently on serotonin. MAO is found both within the synaptic terminals, where it degrades any excess transmitter, and in the liver, where it scavenges circulating catecholamines.

A second enzyme, catechol-O-methyltransferase (COMT), inactivates catecholamines by attaching a methyl group to the 3-OH group of the catechol moiety (Figure 10.3). This is illustrated here only for norepinephrine but happens in just the same way for the other catecholamines as well. Since both MAO and COMT have fairly loose substrate specificities, they can operate on each other's products and hence in either sequence. Before elimination, the MAO/COMT-generated products may or may not be further conjugated by sulfation or glucuronidation, by the same enzymes that also conjugate drug molecules.

10.2. Pharmacokinetic aspects

All three catecholamines are rather polar, due to both the hydroxyl groups and the amino group, which will be mostly protonated at physiological pH. They will thus not cross the blood brain barrier easily, so that in effect the catecholamine pools in the brain and in the periphery will not interfere with each other. Exclusion by the blood brain barrier also applies to many synthetic agonists and antagonists that act on catecholamine receptors, as these compounds often are structurally similar to the physiological mediators. These drugs are useful to influence the peripheral

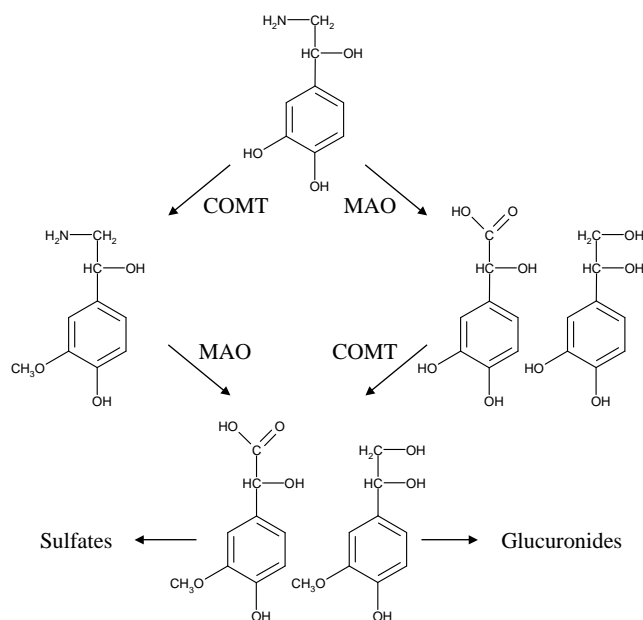


Figure 10.3. Degradation of noradrenaline. COMT, Catechol-O-Methyltransferase; MAO, and monoamine oxidase. MAO initially forms an aldehyde (not shown) that is either reduced to an alcohol or oxidized to a carboxylic acid. Degradation of other catecholamines is analogous.

nervous system (mostly for the purpose of blood pressure regulation) without affecting the central nervous system too much. Others, however, have been especially designed to cross the blood brain barrier. These agents will still be present in high concentration outside the central nervous system and therefore tend to also affect the peripheral functions such as blood pressure and heart rate.

10.3. Drug targets in catecholaminergic synapses

In an catecholaminergic synapse, we find several sites that can be targets of drug action (Figure 10.4):

1. The most straightforward one is, of course, the postsynaptic receptor, to which both agonists and antagonists will bind. As we have seen before, these receptors occur in various types and subtypes. With the three physiological catecholamines, there is a fairly clear distinction between the α - and β -receptors on one hand, which respond to epinephrine and norepinephrine but not dopamine, and the dopaminergic receptors. The major functional difference between epinephrine and norepinephrine consists in their activity on the β_2 receptor subtype, which is very sensitive to the former but not the latter. Synthetic agonists and antagonists (which may or may not closely resemble the natural catecholamines in structure) often have superior type and subtype selectivity, which is both theoretically interesting and useful in therapeutic applications.
2. The presynaptic feedback receptor. In noradrenergic synapses, this is the α_2 receptor. In dopaminergic synapses, the main inhibitory receptor is the D_2 receptor.

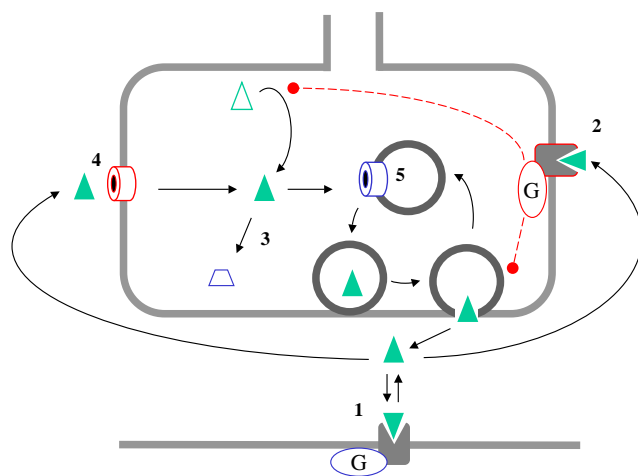


Figure 10.4. Targets of drug action in catecholaminergic synapses. 1 and 2, the post- and presynaptic receptors; 3, breakdown, in particular monoamine oxidase; 4, the presynaptic transmitter reuptake membrane transporters; 5, the vesicular storage transporters.

Both α_2 and D_2 receptors also occur postsynaptically, so their function is not limited to presynaptic inhibition¹.

3. The degradative pathway. Inhibitors of MAO were among the first drugs used in both anti-hypertensive and psychiatric therapy. Though effective, they are not used very much today, for reasons that will become apparent below. Inhibitors of COMT are used in combination with L-DOPA in the therapy of Parkinson's disease (see below).
4. The reuptake membrane transporter in the cytoplasmic membrane. The transporters found in serotonergic, dopaminergic and noradrenergic synapses are highly homologous to one another, and they are somewhat promiscuous with respect to their substrates (e.g., the norepinephrine transporter will transport dopamine with appreciable speed, and vice versa). This also applies to several inhibitors of reuptake, e.g. cocaine, which inhibits the reuptake of dopamine, norepinephrine, and serotonin (see below). To a lesser degree, the homology also applies to the reuptake transporters for glutamate, GABA and glycine, which suggests the possibility of even wider-ranging drug effects.
5. The vesicular transmitter transporter. This transporter is structurally and functionally different from the transporter in the cytoplasmic membrane and accordingly affected by different drugs and mechanisms of drug action.

10.4. Adrenergic receptor agonists and antagonists

The first adrenergic receptor types to be distinguished from each other were the adrenergic α - and β -receptors. Initially based on the different cardiovascular effects of epinephrine and norepinephrine, this distinction was borne out more clearly with the synthetic β -selective agent isoproterenol. Furthermore, subtypes of both α - and β -receptors can be distinguished by selective agonists (Figure 10.5).

10.4.1. Physiological effects of α - and β -selective adrenergic agonists

The different cardiovascular effects of α - and β -receptors are illustrated in the experiment in Figure 10.6. Phenylephrine, an α -selective agent, causes vasoconstriction and

¹ Postsynaptic α_2 -receptors are involved in suppression of pain perception, and their stimulation with the α_2 agonist clonidine is used therapeutically. Some data even suggest that postsynaptic effects are also important in the blood pressure-reducing effects of α_2 agonists. E.g., contrary to what one would expect from the textbook model (which ascribes the antihypertensive effect to presynaptic inhibition), the effect of clonidine persists after ablation of the presynaptic nerve terminals with 6-hydroxydopamine (see below).

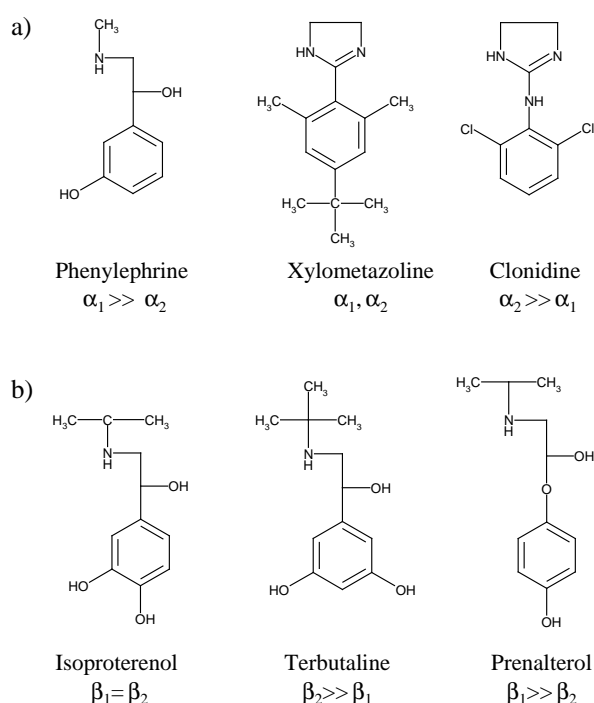


Figure 10.5. Structures and subtype specificities of selected adrenergic agonists. a, α -selective agonists; b, β -selective agonists.

accordingly a rise in blood pressure. The heart has few α -receptors, so phenylephrine does not accelerate the heart rate. Rather, the heart rate drops a little, due to an autonomic physiological reflex triggered by the increased blood pressure². Conversely, the β -selective agonist isoproterenol speeds up the heart rate quite significantly, yet nevertheless causes the blood pressure to drop, which is due to vasodilatation. The initial brief spike of the blood pressure is probably caused by delayed distribution of the drug into the skeletal muscle, where most of the vascular β_2 -receptors are located. Vasodilatation will therefore lag behind the stimulatory effect on the heart, since the heart is perfused much more strongly than the skeletal muscle, especially at rest. Epinephrine, which stimulates both α - and β -receptors, shows an intermediate response.

10.4.2. Physiological effects of α_2 -adrenergic agonists

The vasopressor response seen with phenylephrine is particularly strong because it is an α_1 -selective agonist. α_2 -Agonists actually lower the blood pressure, which is in accord with the role of the α_2 -receptors in presynaptic inhibition. This inhibition is shown in Figure 10.7a: The re-

² Note that the dog used in this experiment had been anaesthetized. Narcosis will weaken autonomic physiological reflexes to the agents. E.g., with phenylephrine and without narcosis, the rise in blood pressure might be less pronounced due to a more pronounced reflexory drop in the heart rate.

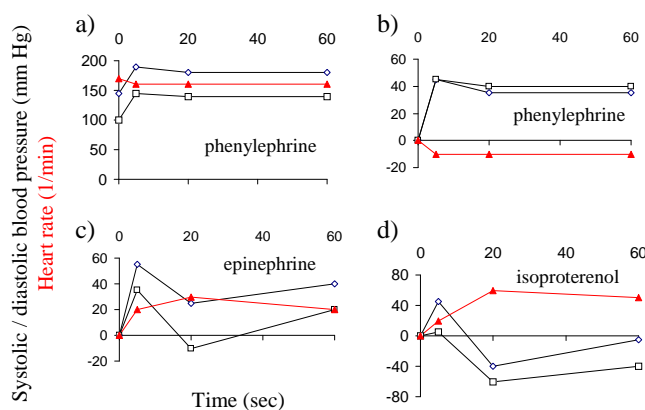


Figure 10.6. Effects of adrenergic agonists on the heart rate (▲) and the systolic³ (◇) and diastolic (□) blood pressure in an anesthetized dog. a: Absolute values after application of phenylephrine; b, c, d, differences relative to time zero after application of the respective drugs.

lease of radioactively labeled norepinephrine from the nerve terminals is reduced by about 80% with an α_2 agonist (UK 14,304). In the presence of α -receptor blockers (rauwolscine or phentolamine), we see a right shift of the dose response curve, which confirms the receptor-specific mode of action of this effect.

Further confirmation of the role of the α_2 -receptor comes from gene knockout experiments (Figure 10.7b). There are actually three discernible subtypes (or subsubtypes – have as many subs as you please) of the α_2 receptor, named A/D, B and C, with distinct genes on different chromosomes. In the particular cell type used in the experiment shown, the A/D subtype was evidently responsible for most of the inhibition of transmitter release.

While this inhibition is very nice and unequivocal, the intracellular events that occur subsequent to α_2 receptor stimulation are less clearly defined. The experiment depicted in Figure 10.7c suggests that indeed, as stated earlier, the α_2 -receptor acts by lowering cAMP. Pertussis toxin – the major toxin secreted by the bacterium *Bordetella pertussis*, the causative agent of whooping cough – is able to cross the cell membrane and then covalently modify⁴ the α -subunit of the G_i protein that normally inhibits adenylate cyclase when activated by the α_2 receptor.

Although pertussis toxin is a popular tool in signal transduction research, it acts on several G proteins other than G_i as well, so that the implication of cAMP by toxin inhibition is

³ The blood pressure changes periodically with the heart beat. The 'systolic' value is the maximum, and the diastolic value the minimum observed pressure in each period.

⁴ By ADP-ribosylation: NAD is cleaved, and its ADP-ribosyl moiety is attached to strategic site of the protein molecule. This is a very common mode of action of bacterial toxins that applies to a wide variety of target proteins in the eukaryotic cell.

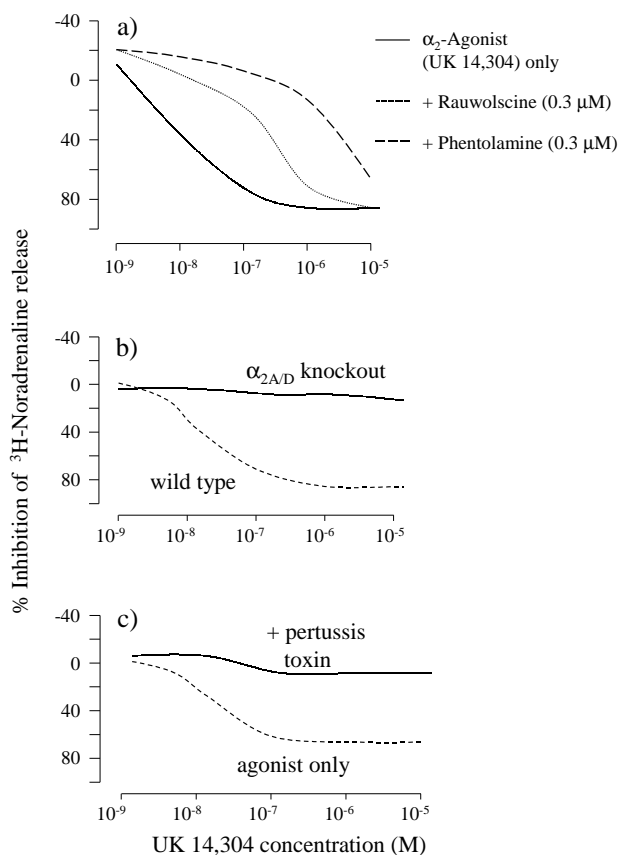


Figure 10.7. Role of the α_2 -adrenergic receptor in presynaptic regulation of norepinephrine release. a: Presynaptic inhibition of norepinephrine release by the experimental α_2 -receptor agonist UK 14,304, and inhibition of this inhibition by the α -receptor antagonists rauwolscine and phentolamine. b: Abrogation of α_2 -mediated inhibition by genetic knockout of the A/D receptor subtype. c: Abrogation of α_2 -mediated presynaptic inhibition by pertussis toxin. Pertussis toxin ADP-ribosylates $G_{i,\alpha}$ and thereby prevents its inhibitory effect on adenylate cyclase. Data from: *Naunyn Schmiedeberg's Arch. Pharmacol.* 363:110-9, 2001.

not cogent. In fact, experimental evidence is also available that throws the role of cAMP in α_2 -mediated effects into question. You will recall that the release of insulin from pancreatic islet cells is closely similar to neurotransmitter exocytosis. It is stimulated by glucose and further amplified by cAMP. The effect of cAMP can be studied using the cell-permeant synthetic analog dibutyryl-cAMP (Figure 10.8a). Intracellularly, the butyryl and acetyl groups of this 'pro-drug' will get cleaved off to release cAMP itself, and in Figure 10.8b you can see that the cAMP released indeed increases insulin secretion. However, at sufficiently high concentrations, clonidine suppresses the increased insulin release caused by cAMP (Figure 10.8c). This suggests that the effect of the α_2 -receptor stimulation must be located somewhere downstream of adenylate cyclase and cAMP. α_2 -Receptor stimulation has measurable effects on Ca^{++} and K^+ channels, which are involved in insulin secretion.

How these effects relate to cAMP or to the $\beta\gamma$ -subunits of G_i is not known with certainty.

Figure 10.9 illustrates the blood-pressure lowering effect of clonidine. As with isoproterenol (cf. Figure 10.6), the early effects go into the opposite direction, but here this phase lasts minutes rather than seconds, which probably reflects the need of the drug to cross the blood brain barrier to elicit the drop in blood pressure. In this case, we see a very strong reflexory increase of the heart rate that finally negates the drop in blood pressure. This type of counter-regulation is very common. Effective treatment of hypertension therefore frequently requires the combined use of several drugs with complementary modes of action. For example, the reflexory increase in heart rate could effectively be countered by the simultaneous application of β -receptor antagonists.

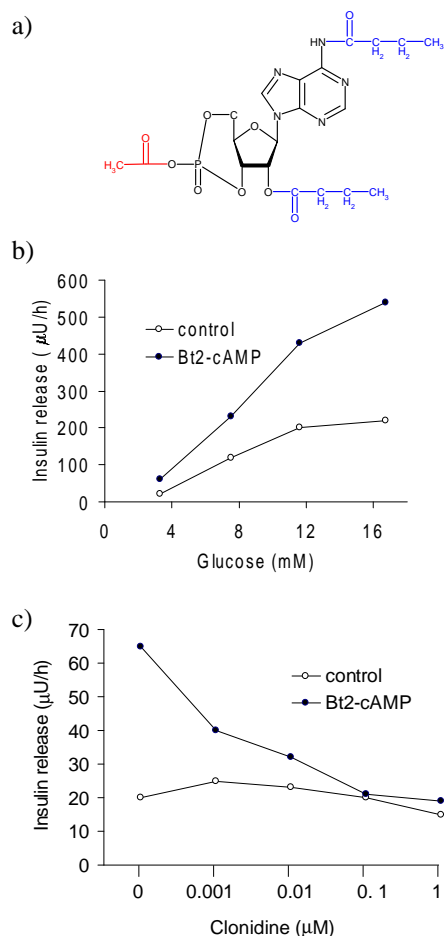


Figure 10.8. Effect of cAMP on insulin secretion in pancreatic islet cells. a: Structure of the membrane-permeant analog dibutyl-cAMP. The two butyryl groups and the acetyl group will be cleaved intracellularly, releasing cAMP. b: Glucose-dependent insulin secretion in the presence or absence of dibutyl-cAMP. cAMP amplifies glucose-dependent secretion. c: Suppression of cAMP-mediated stimulation of insulin secretion by the α_2 -agonist clonidine. (Data from *Life Sci.* 32:191-95, 1983.)

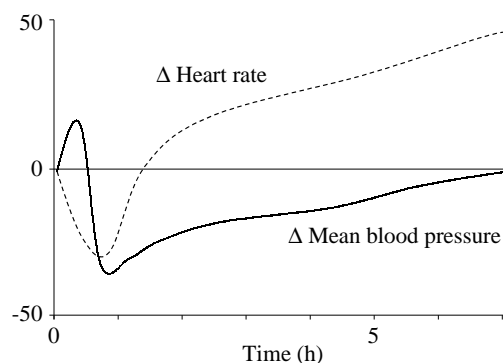


Figure 10.9. Effect of intravenous clonidine upon heart rate and mean blood pressure (after data from: *Journal of Pharmacology And Experimental Therapeutics* 306:271-8).

10.4.3. β -Adrenergic agonists

β_1 -selective agonists such as prenalterol (Figure 10.5) or dobutamine are used to stimulate heart contractility and heart rate in patients that exhibit too low blood pressure. β_2 -Selective agents such as terbutaline find application for relaxation and dilatation of the bronchi in the treatment of asthma or chronic obstructive lung disease, and in the suppression of premature labour. The mechanism of β -receptor-induced relaxation of smooth muscle – cAMP-mediated phosphorylation and inhibition of myosin light chain kinase – has been described before.

10.4.4. α -Adrenergic antagonists

Like agonists, adrenergic receptor antagonists come in various degrees of type and subtype selectivity. Among the α -receptor antagonists, phentolamine and tolazoline (Figure 10.10) act on both α_1 - and α_2 -receptors. α_2 -Selective antagonists do exist (e.g. yohimbine) but don't have major therapeutical applications. Considering the physiological role of α_2 receptors, it is clear that α_1 -selective antagonists such as prazosine are preferable for use in the therapy of hypertension. In addition to reversible blockers, irreversible (covalent) blockers are available for particular situations such as adrenaline-producing tumours (cf. the discussion of phenoxybenzamine in the chapter on pharmacodynamics).

10.4.5. β -Adrenergic antagonists

The practically most important class of drugs directly acting on any adrenergic receptor are the β -blockers (Figure 10.12). They are mainly used to reduce the workload on a heart subject to impaired perfusion due to atherosclerotic blood vessel obliteration. The most severe consequence of this impaired perfusion is myocardial infarction, which consists in the irreversible damage to the regions of heart muscle tissue downstream of an obliterated artery. Patients

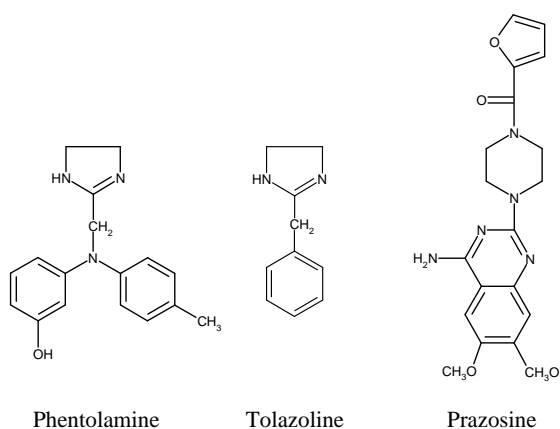


Figure 10.10. Structures of α -receptor blockers. Phentolamine and tolazoline block both α_1 - and α_2 -receptors, whereas prazosine selectively inhibits α_1 -receptors.

experiencing the first such event are at considerable continuous risk of repeated attacks. Accordingly, the number of survivors in this group of patients decreases substantially with time. β -Blockers are among the very few agents that afford them a substantial and indubitable benefit, in terms of life expectancy. While in Figure 10.12b atenolol appears somewhat superior to propranolol, the effect of another β_2 -selective blocker (metoprolol) was virtually indistinguishable from that of propranolol (not shown). There is thus far no conclusive evidence that β_2 -selective antagonists really are more effective than those that are not subtype-specific.

10.5. Inhibitors of presynaptic transmitter reuptake

Presynaptic reuptake of catecholamines works by cotransport of sodium and chloride ions. While the reuptake transporters are homologous among the various types of catecholaminergic and serotonergic synapses, the stoichiometry of transmitter molecules and co-transported ions appears to vary, as depicted in Figure 10.11a. The transport process is facilitated both by ion concentration gradients and by the resting membrane potential (note that reuptake will cause a net transport of positive charge to the cytosol). Inhibition of presynaptic transmitter reuptake is another very important principle of drug action at adrenergic and serotonergic synapses. It will have several consequences (Figure 10.11b):

1. It will increase the concentration of transmitter in the synaptic cleft and, therefore, the postsynaptic stimulatory action.
2. The postsynaptic cells will respond with a reduction of receptors exposed on the surface. This is one of the effects leading to a fairly rapid and noticeable decrease

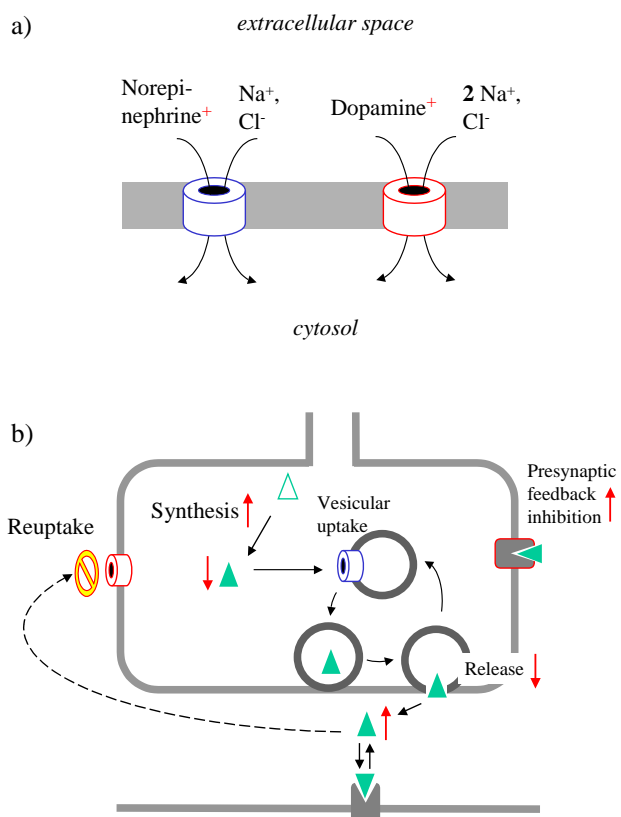


Figure 10.11. Presynaptic reuptake of norepinephrine and dopamine by sodium and chloride cotransport (a), and functional consequences of inhibition of reuptake (b).

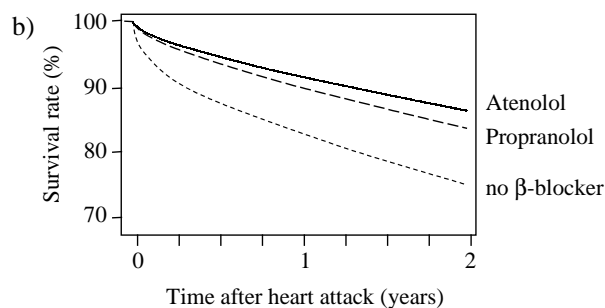
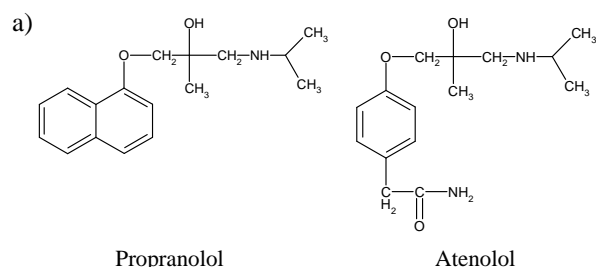


Figure 10.12. β -Receptor blockers. a: Structures of propranolol (not subtype-selective) and of atenolol (β_2 -selective). b: Effect of propranolol and of atenolol on the survival rate of patients after myocardial infarction. Data from *American Journal of Cardiology*, 87:823-826 (2001)

in drug efficacy, and to habituation and possibly addiction.

3. It will increase the presynaptic negative feedback, reducing both the rate of transmitter release and (by regulation at the genetic level) the amount of synthetic enzymes, e.g. of tyrosine hydroxylase.
4. The intracellular pool of transmitter will be depleted. This will disinhibit tyrosine hydroxylase at the protein level, and therefore the actual turnover of synthesis will be increased, despite the reduced prevalence of synthetic enzymes.

Again, there are various drugs with different ranges and specificities. Cocaine has a particularly broad spectrum, affecting the reuptake of norepinephrine, dopamine, and serotonin alike. The effect of cocaine can be quantitatively studied in mice by observing their excitement in response to being placed into a new environment, which is measured simply as the distance travelled within their new home over time. In experiments with transgenic mice, the reuptake transporters for both dopamine and serotonin had to be deleted in order to abolish the increase in excitement induced by cocaine.

In humans, a prominent effect of cocaine consists in increased vigilance and elevated mood. While cocaine itself is not used clinically, several catecholamine and serotonin reuptake blockers are used as antidepressants. Imipramine (Figure 10.13) is a 'classic' but not so very specific; in addition to inhibiting the reuptake of serotonin and of norepinephrine, it also has antihistaminic and antimuscarinic⁵ activity. This will lead to side effects in both the central nervous system and the peripheral autonomic system. A prominent one is the causation or deterioration of cardiac arrhythmias due to its antimuscarinic action.

Imipramine and its many structurally similar congeners are collectively referred to as the 'tricyclic antidepressants'. A more modern drug is fluoxetine, which has similar antidepressant action but lacks most of the side effects of imipramine. Its selectivity for serotonin reuptake supports the notion that this transmitter, indeed, has a major role in regulating mood.

Since inhibition of transmitter reuptake depletes both the presynaptic transmitter and the postsynaptic receptors, it is clear that immediately after sudden discontinuation of a reuptake inhibitor the level of postsynaptic excitation would be very much reduced. Therefore, termination of antidepressant therapy must always be done carefully and slowly.

⁵This activity is responsible for one of its major side effects - disturbing the heart rhythm.

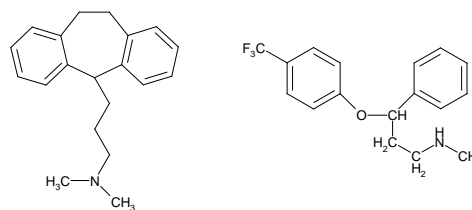


Figure 10.13. Structures and action profiles of imipramine (left) and fluoxetine, two representative reuptake inhibitors that are used clinically as antidepressants.

10.6. Inhibition of vesicular storage

Vesicular accumulation of catecholamines and of serotonin is inhibited by reserpine (Figure 10.14a). While reserpine initially was believed to inhibit the H^+ -ATPase that generates and maintains a high proton concentration inside the vesicles⁶, it is now clear that reserpine instead binds to the vesicular transmitter transporter that makes use of this proton gradient to move the transmitter uphill its own gradient into the vesicle (Figure 10.14b). The number of protons released for the import of each transmitter molecule is not known with certainty but is likely greater than 1.

Reserpine affects both the central nervous system⁷ and the peripheral autonomic system. The immediate effect will be the accumulation of transmitter in the cytosol. From there, it may 'spill over', possibly by retrograde operation of the specific reuptake transporters, into the synaptic cleft. Accordingly, a transient 'sympathomimetic' effect may be seen after application of a high dosage of reserpine. However, the cytosolic transmitter excess will soon be scavenged by increased breakdown and inhibition of synthesis, and the main effect of inhibited synaptic transmission will become manifest (Figure 10.14c). The action of reserpine was initially observed in an extract of the Indian plant *Rauwolfia serpentina*, which was noticed to have antipsychotic effects⁸. After it was purified, the drug was used initially in the same application but later on was mainly used for anti-hypertensive treatment. It now has largely been abandoned in favour of more selective agents.

⁶Interestingly, reserpine does inhibit some types of ATP-dependent drug extrusion transporters, both in eukaryotic and bacterial cells. Bacteriologists have become interested in this effect, since drug extrusion is an important means of bacterial resistance to antibiotics.

⁷Reserpine is a rather large molecule, and one might not expect it to cross the blood brain barrier easily. However, it binds to its targets with very high affinity and virtually irreversibly, so that even small concentrations in the brain may suffice to elicit noticeable effects.

⁸By analogy to modern agents, I would guess that the patients responding favourably were suffering from mania or schizophrenia rather than depression. In fact, one common side effect of reserpine (in patients receiving it as a treatment of hypertension) consists in its triggering of depressive mood.

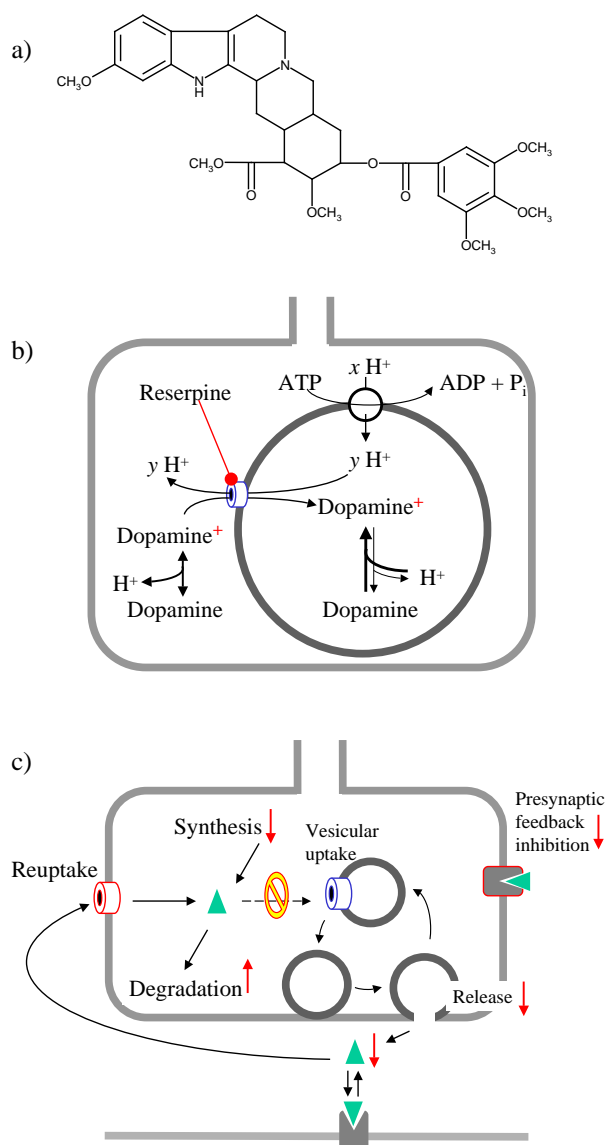


Figure 10.14. Structure (a) and mode of action of reserpine. b: Reserpine inhibits vesicular accumulation of dopamine and norepinephrine by blocking the vesicular proton antiporter. c: Functional consequences of reserpine action. The net effect consists in reduced activity of catecholaminergic synapses.

10.7. Indirect sympathomimetics

A more complex (and still somewhat contentious) mechanism of action is found with a group of drugs known as ‘indirect sympathomimetics’. Example structures are shown in Figure 10.15a. As you can see, all these structures lack the phenolic –OH groups present in epinephrine. This prevents their modification by catechol-O-methyltransferase and slows down their deamination by monoamine oxidase. It also facilitates their crossing of the blood brain barrier, and it is mostly for their central effects – enhanced vigilance, elation, and suppression of appetite, resulting in weight loss – that these drugs have been used and abused. Amphetamine and methamphetamine preferen-

tially act on catecholaminergic synapses, in particular on those containing norepinephrine. In contrast, the substance 3,4-methylenedioxymphetamine (a.k.a. ‘ecstasy’; Figure 10.15b) has a stronger effect on serotonergic synapses. It is likewise popular as a drug of abuse.

Amphetamine acts on both transmitter transporters – the plasmalemmal one, which is the site of action of cocaine, and the vesicular transporter, which is targeted by reserpine. It is imported into the cell by the plasmalemmal transporter. This will result in inhibited reuptake of the physiological transmitter, not so much apparently by direct competition (as is the case with cocaine) but by subsequent endocytosis of the receptor. This is clearly shown in Figure 10.16. In the experiment shown, the dopamine reuptake transporter was recombinantly expressed in cultured cells and visualized by immunofluorescence⁹. Initially, the fluorescence is confined to the surface of the cells expressing the transporter¹⁰ (Figure 10.16b, left panel). After exposure of the cells to amphetamine, the stain gradually disappears from the surface and is translocated into the cell interior, indicating endocytosis of the transporter¹¹. Simultaneously, the

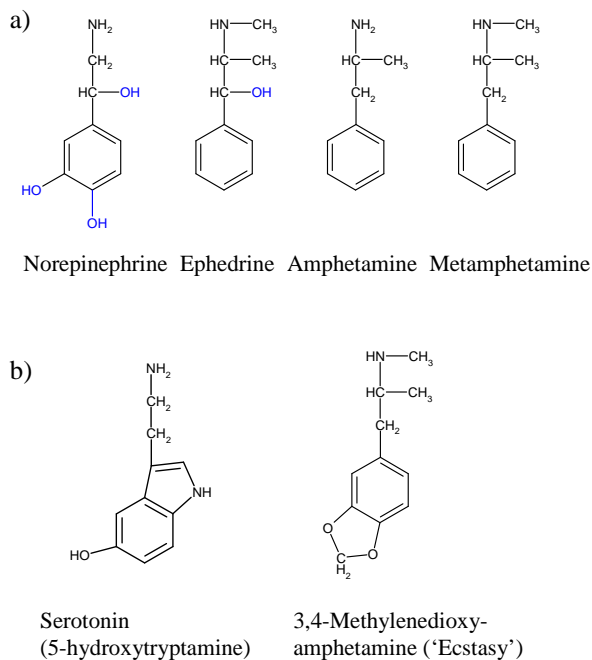


Figure 10.15. Structures of several indirect sympathomimetics (a), and of the functionally similar drug ‘ecstasy’ (b). Norepinephrine and serotonin are shown for comparison.

⁹ Antibodies that specifically bind the target molecule are visualized by fluorescence, usually with a fluorescein-labeled secondary antibody against the (unlabeled) first one.

¹⁰ Expression is recombinant and in cell culture, and the cells lack the typical aspect of neurons. The transporter appears to be uniformly distributed over the entire cell surface but not confined to specialized regions as in the case of nerve terminals.

¹¹ Endocytosis is a fast and usually reversible means of regulating the

transport capacity for dopamine drops by some 80-90%, as measured by the current across the cell membrane induced by dopamine (Figure 10.16a). The transport can be measured as a current, since for each molecule of dopamine taken up there is a net transfer of 2 cations into the cytoplasm (cf. Figure 10.11a; note that dopamine itself carries a positive charge, too). There is not much known about how amphetamine import triggers transporter endocytosis.

In addition to interfering with the reuptake of catecholamine transmitters, amphetamine will also release transmitter that is stored inside vesicles. This is different from reserpine, which only prevents uptake of more transmitter molecules into the vesicles but does not cause release of those already inside. The release itself can be demonstrated quite clearly with transmitter vesicles isolated from nerve tissue (Figure 10.17a); its mechanism, however, is still contentious. From several reports in the literature, I have distilled the model¹² depicted in Figure 10.17b.

As with the plasmalemmal transporter, the ionized form of the transmitter is the one that is the substrate for active transport into the vesicle. We saw above that this transport is powered by antiport of protons from the vesicle into the

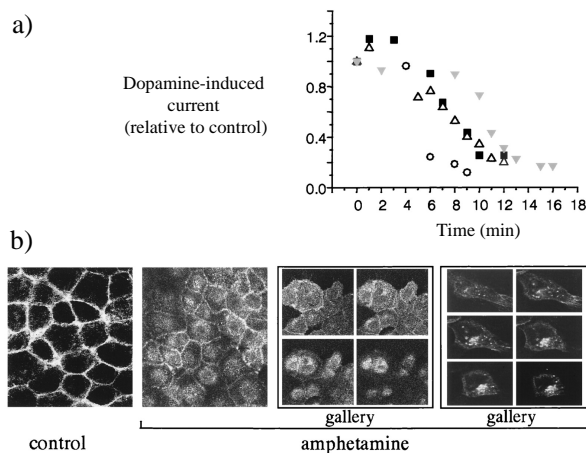


Figure 10.16. Endocytosis of dopamine reuptake transporters triggered by amphetamine. a: Application of amphetamine reduces the transmembrane current that is a measure of dopamine reuptake. Shown are several independent experiments. b: Detection of the transporter by immunofluorescence and confocal microscopy. In the absence of amphetamine, the transporter is located at the cell surfaces. Amphetamine induces translocation of the fluorescence to intracellular compartments.

activity of surface receptors and transporters. Other frequently cited examples are the β -adrenergic receptor, and the insulin-dependent glucose transporter.

¹² Illustrated here for dopamine, but intended to apply to other catecholamines alike. This model is in accord with all empirical data I saw, yet I did not review all there are, and so it may or may not be true. Also, it may be found elsewhere in the literature; however, I did not see it in any of those papers I read about this topic.

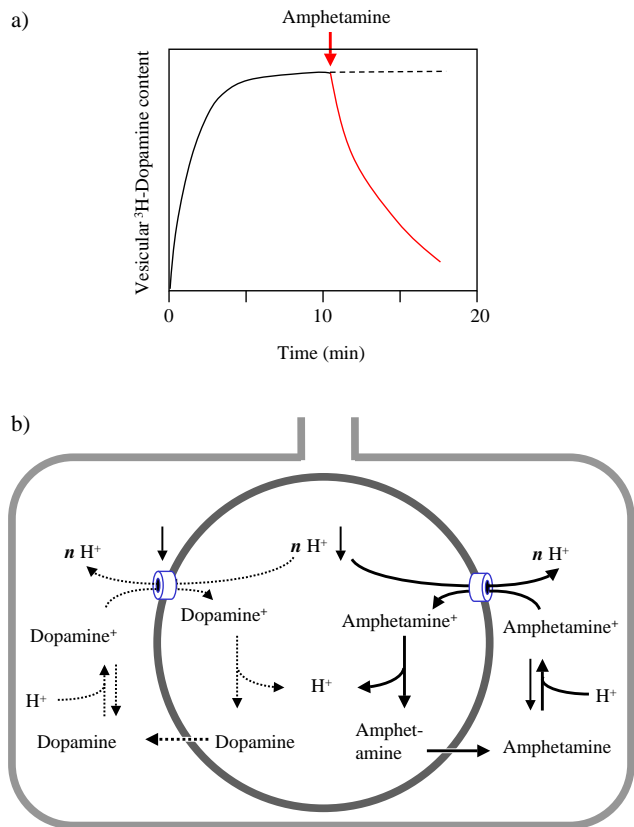


Figure 10.17. Depletion of dopamine from neurotransmitter vesicles. a: Vesicular accumulation and retention of dopamine can be experimentally observed by bathing isolated vesicles in ³H-Dopamine. Upon addition of amphetamine, the ³H-dopamine is swiftly released. b: Hypothetical mechanism. Amphetamine induces a futile cycle of transport which depletes intravesicular protons and thereby reduces vesicular uptake of dopamine and also promotes dopamine leakage by non-ionic diffusion.

cytosol. For the hypothetical mechanism of transmitter depletion to work, it is important that there be more than one proton released from the vesicle in exchange for every catecholamine molecule imported (i.e., $n > 1$ in Figure 10.17b).

The high proton concentration in the vesicle serves yet another purpose, which consists in trapping the catecholamine in its protonated form inside the vesicle (and thereby preventing it from slipping back across the membrane by non-ionic diffusion). Amphetamine would derail this mechanism by again functioning as a substrate for transport. This would lead to depletion of the protons from the vesicle interior. Both amphetamine and the transmitter itself would thus be present to an increased extent in their non-protonated forms and thus would leak out of the vesicle by non-ionic diffusion. Amphetamine, in particular, being less polar, would efficiently escape from the vesicle and thus by futile cycling between cytosol and vesicle exhaust the capacities of the proton pump and, possibly, the transporter.

10.8. L-DOPA and carbidopa in the therapy of Parkinson's disease

Like the transporters for the catecholamines and serotonin, those for their precursor amino acids are not of very high specificity. This has been exploited in various ways for pharmacotherapy. A very important example is the use of L-DOPA as a pre-drug to substitute dopamine to the brain in patients with Parkinson's disease (Figure 10.18a). Dopamine itself cannot cross the blood brain barrier¹³. However, L-DOPA is accepted by an amino acid carrier that normally transports aromatic amino acids. It can thus enter the brain and there be decarboxylated to dopamine.

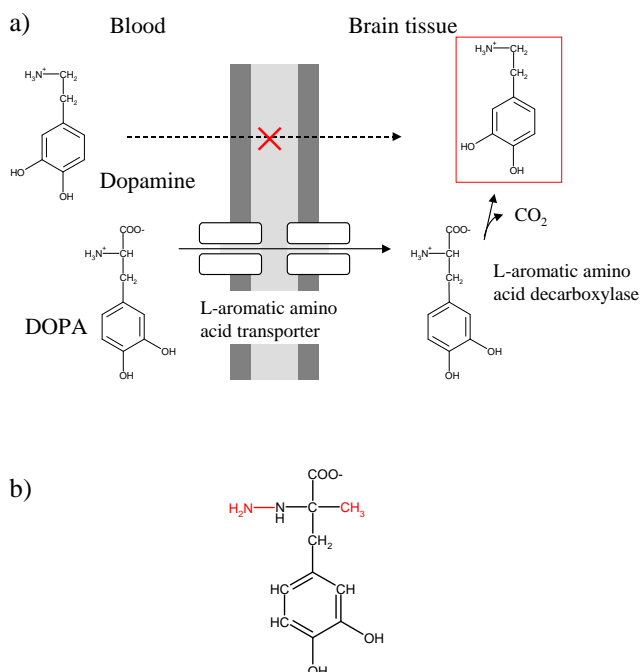


Figure 10.18. DOPA in the therapy of Parkinson's disease. a: Transport of DOPA across the blood brain barrier by the aromatic amino acid transporter, and its intracerebral decarboxylation to dopamine. b: Structure of carbidopa, an inhibitor of DOPA decarboxylase. Carbidopa does not cross the blood brain barrier and therefore selectively inhibits the degradation of DOPA in the periphery, permitting substantial reduction of therapeutic DOPA dosages.

Concurrently with its permeation into the brain, however, DOPA will also be decarboxylated in the periphery to dopamine; thus, the overall fraction of DOPA that winds

up in the brain is only about 2%. This means that very high dosages will be required for the desired clinical effect, and the periphery would be uselessly troubled with high amounts of dopamine and derived catecholamines. This situation can be greatly improved by the simultaneous application of an inhibitor of DOPA decarboxylase, called carbidopa (Figure 10.18b). Note that this substance will not cross the blood brain barrier, and therefore not interfere with the (necessary) decarboxylation in the brain.

Note that, since dopamine is the precursor of norepinephrine and epinephrine, carbidopa will inhibit the synthesis of all three catecholamines. One therefore might expect it to reduce blood pressure, but in animal experiments it rather seems to increase it; this has been attributed to the lack of dopamine in the periphery.

10.9. 'False transmitters'

Another drug closely similar to DOPA but used for different applications is α -methyl-DOPA (Figure 10.19a). This molecule acts in the peripheral autonomous system but also enters the brain, by the same route as DOPA. It is converted by DOPA decarboxylase to the 'false transmitter' α -methyl-dopamine. Like dopamine or norepinephrine, α -methyl-dopamine is accumulated inside the transmitter vesicles, and released in response to action potentials. While it has no strong effect on postsynaptic α_1 -receptors, it does activate α_2 -receptors. It will therefore inhibit the further release of transmitter without stimulating the postsynaptic neuron. The effect of methyl-DOPA is augmented by the fact that it is fairly resistant to monoamine oxidase. Its mode of action resembles that of clonidine (which accomplishes the same in a less roundabout manner).

Another example of a 'false transmitter' is the drug guanethidine (Figure 10.19b). This drug is quite different in structure from methyl-DOPA. It is therefore not piggy-backed across the blood brain barrier by the aromatic amino acid transporter (nor by any other specific transporter), and since it is polar it does not cross by simple diffusion either. However, it may enter post-ganglionic sympathetic neurons in the periphery by way of reuptake transporters. Accordingly, guanethidine can lower blood pressure by a purely peripheral mechanism.

10.10. Cytotoxic catecholamine analogs

Like DOPA and methyl-dopa, 6-hydroxydopa (Figure 10.20a) finds its way into catecholaminergic nerve terminals; it is decarboxylated inside the cell to 6-hydroxydopamine. The latter compound, however, does not act as a false transmitter. Instead, it acts as an inhibitor of the mitochondrial respiratory chain, apparently by binding to com-

¹³If you have followed closely what has been said before, you might now be wondering why dopamine should be too polar to cross the membranes that represent the blood brain barrier, but should readily cross those of the presynaptic vesicles. The answer to this apparent paradox (aside from the fact that the blood brain barrier actually consists of four membranes in series) lies in the vastly different surface-to-volume ratios of the two compartments. Think of a pinhole in a thimble vs. one in a swimming pool.

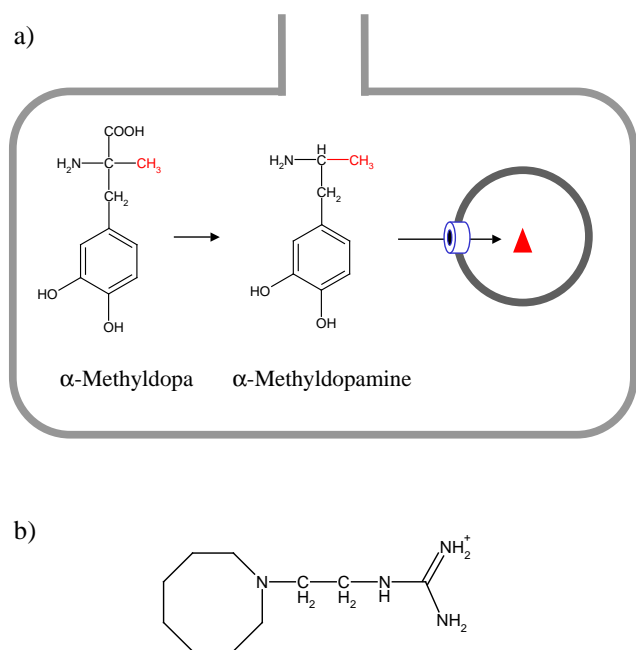


Figure 10.19. ‘False transmitters’ that are used for antihypertensive treatment. a: α -Methyldopa passes the blood brain barrier and is decarboxylated to α -methyldopamine, which upon synaptic release stimulates presynaptic α_2 -receptors. b: Guanethidine acts on catecholaminergic synapses in the peripheral autonomous nervous system.

plex I (NADH dehydrogenase). This leads to degeneration of the norepinephrine- or dopaminergic neurons. When applied to newborn rats, it largely destroys the sympathetic nervous system (both its central and peripheral parts), and it is being used for this purpose in experimental research.

A drug with a similar mode of action is Methyl-phenyl-tetrahydropyridine (MPTP; Figure 10.20b). This substance is converted (apparently by monoamine oxidase, which also oxidizes the regular catecholamine transmitters) to 1-Methyl-4-phenyl-pyridinium (MPP⁺). MPP⁺ is again an inhibitor of mitochondrial NADH dehydrogenase. Since MPTP enters the neurons through the dopamine reuptake transporter, it acts selectively on dopaminergic cells and, accordingly, gives rise to a drug-induced form of Parkinson’s disease. Of course, MPTP is not being used in clinical medicine. It is formed as a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP, Figure 10.20b), which in turn has a morphine-like mode of action. The poisonous action of MPTP was discovered accidentally when drug addicts suddenly developed the symptoms of Morbus Parkinson after partaking of a batch of MPPP from an illegally (and, it would seem, unprofessionally) operated laboratory that contained an exceptionally high proportion of MPTP. MPTP has, however, subsequently found application in experimental research on the latter disease with animals.

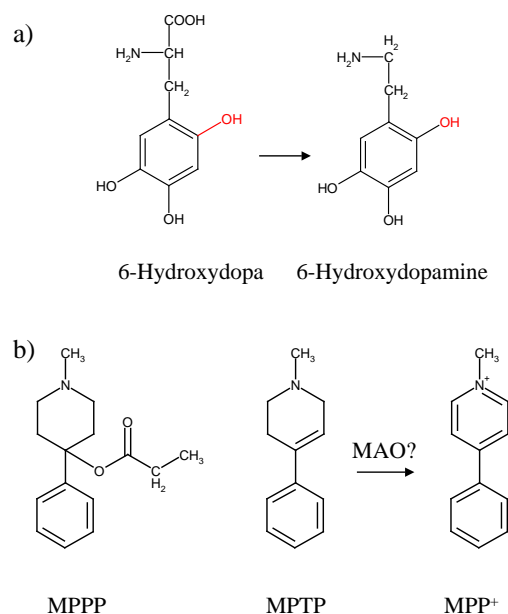


Figure 10.20. Structures of cytotoxic catecholamine analogues. a: 6-Hydroxydopa is converted to the cytotoxic metabolite 6-hydroxydopamine. b: Methyl-phenyl-tetrahydropyridine (MPTP) is formed as a byproduct of the synthetic opioid 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP). It is converted to the cytotoxic metabolite methyl-phenyl-pyridinium, probably by monoamine oxidase.

10.11. Monoamine oxidase inhibitors

The last class of drugs we will consider are the inhibitors of monoamine oxidase (MAO). When we compare the reaction products in Figure 10.3 and in Figure 10.20b, respectively, they look fairly different¹⁴; yet both may be accounted for by the enzymatic mechanism outlined in Figure 10.21a in simplified form¹⁵. The enzyme reaction starts with the abstraction of an electron from the substrate, which converts both the substrate and the enzyme to radicals. Subsequently, the substrate is dehydrogenated to a Schiff Base, which in turn is hydrolyzed to an aldehyde. Hydrogen and electrons wind up bound to the FAD prosthetic group of the enzyme.

The hypothetical reaction mechanism for the inhibitor tranylcypromine is shown in Figure 10.21b. Abstraction of the first electron causes the (instable) cyclopropyl ring to open, and the radical thus formed recombines with the one formed at the enzyme to yield a covalent adduct. Because

¹⁴The question whether or not MPP⁺ is actually generated by monoamine oxidase seems to be unsettled. Not being an expert on this or on enzyme mechanisms in general, I think MPP⁺ might indeed be formed by repeated cycles of oxidation (four hydrogen atoms and one electron total are abstracted from MPTP, whereas only two hydrogens are abstracted from a catecholamine substrate that is oxidized to an aldehyde.)

¹⁵While the ultimate electron acceptor in the enzyme molecule is its FAD coenzyme moiety, the radical combination products with inhibitors (cf. Fig. 38) may involve either the FAD or an active site cysteine.

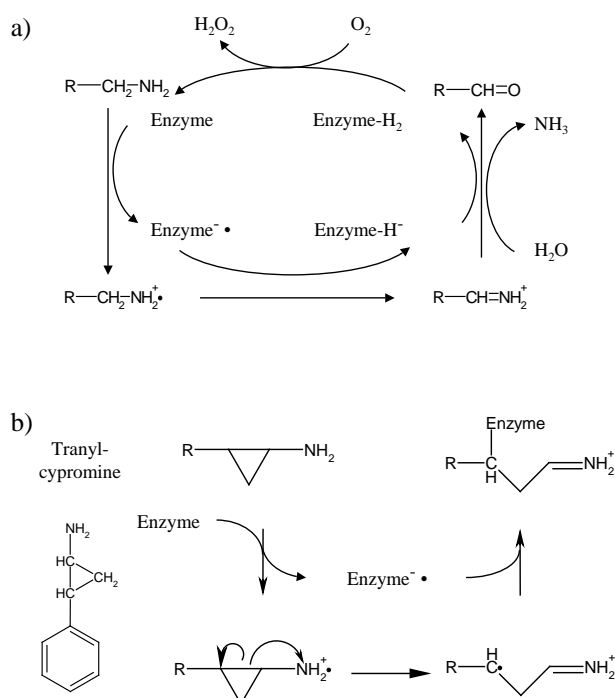


Figure 10.21. Mechanism of monoamine oxidase (a), and its inhibition by tranylcypromine (b). The enzyme goes through a radical intermediate state, which may recombine with a radical conversion product of the inhibitor. Accordingly, the inhibitor becomes bound covalently to the enzyme.

of this covalent attachment to the enzyme, the effect of MAO inhibitors outlasts the elimination of the drug and is only reversed by synthesis of new enzyme, which will require days to weeks after discontinuation.

MAO inhibitors will act peripherally and may act centrally, again depending on their pharmacokinetic properties. They have, like reserpine, been used for both antihypertensive and antipsychotic treatment but now been superseded by more selectively acting drugs. However, there recently has been renewed interest in the development of MAO B-selective inhibitors, since that enzyme subtype acts preferentially on serotonin and in the central nervous system; some of the side effects could thus be avoided or ameliorated. MAO B inhibitors have also been reported to increase the lifetime of dopamine and therefore to be beneficial in Parkinson's disease; similarly, inhibitors of COMT have more recently been introduced as a supplement to therapy in this disease.

Hello, wake up. So, why should MAO inhibitors have an antihypertensive effect? Decreased degradation of catecholamines should increase the availability of norepinephrine and increase rather than decrease blood pressure, shouldn't it? My textbook says that it works as follows (Figure 10.22): Small amounts of tyrosine will always get decarboxylated to tyramine. Normally, tyramine is scavenged by monoamine oxidase. However, if this pathway is blocked, tyramine will get converted instead to oc-

topamine (by dopamine hydroxylase). Octopamine will then act as a false transmitter, in the same way as discussed above for guanethidine and methyl-DOPA.

Nice huh? But most likely wrong. The same text does not fail to mention the so-called 'cheese reaction', which consists in a sudden rise of blood pressure in patients receiving MAO inhibitors. Cheese – as well as other types of fermented food, such as salami or summer sausage – is rich in decarboxylation products of amino acids (amines), which are in part responsible for the characteristic flavours. The one of interest here is indeed tyramine¹⁶. Tyramine acts as an 'indirect sympathomimetic', much in the same way as amphetamine does. It can hardly be held responsible for lowering and increasing the blood pressure at the same time¹⁷.

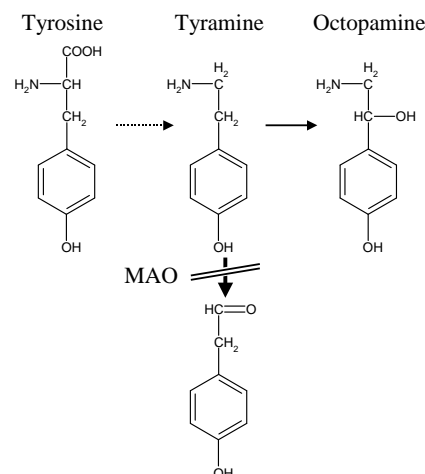


Figure 10.22. Hypothetical mechanism of the antihypertensive effect of monoamine inhibitors. This mechanism does not fit the observation of the 'cheese reaction', in which tyramine contained in fermented food causes hypertensive episodes in patients receiving monoamine oxidase blockers.

¹⁶Tyramine seems to be more prevalent with bacterially fermented foods, and obviously those rich in protein rather than carbohydrates. E.g., wine and beer, which are fermented by yeast, have lower contents of tyramine than sausage and cheese, most of which are bacterially fermented (although Camembert and Roquefort are not).

¹⁷Textbooks often have a deplorable tendency to 'explain' the unexplained – thus preventing rather than stimulating independent thought. For an illustration, grab a really old textbook on whatever subject you like – if there is one thing UW has aplenty, it is outdated textbooks of any description. You may find lots of things 'explained' that were completely unknown at the time.

(Notes)

Chapter 11. Pharmacology of nitric oxide (NO)

Nitric oxide is a mediator that is very different from any other hormones and transmitters. Three key properties of NO are important to its unique mode of signal transmission:

- NO is a very small molecule and permeates cell membranes with ease – its membrane permeability is comparable to that of oxygen.
- It binds very fast and avidly to heme, as both O₂ and CO do as well. Its affinity for heme is higher than that of O₂ but lower than that of CO. Binding of NO to heme is at the heart of its major established signalling mechanism.
- NO is a radical ($\cdot\text{N}=\text{O}$) and therefore quite reactive. It can react with molecular oxygen and various reactive oxygen species. The ensuing products in turn may react with amino acid side chains in proteins, leading to S-nitrosylation of cysteines and O-nitrosylation of tyrosines. The significance of protein nitrosylation in signalling is still a matter of debate; we will look at some experimental data below.

11.1. Vascular effects of nitric oxide

The pharmacological activity of nitric oxide was recognized before it was identified as a physiological mediator itself. This discovery was made during an investigation into the nature of the so-called ‘endothelium-derived relaxing factor’ (EDRF). The activity of EDRF can be triggered by the application of acetylcholine to the aorta of experimental animals. In the aorta (as well as other blood vessels), not only the smooth muscle itself but also the endothelium is supplied with cholinergic nerve terminals and accordingly possesses acetylcholine receptors, which are of the muscarinic type (Figure 11.1a). After cutting the aorta into strips (Figure 11.1b), the endothelium can be removed mechanically or enzymatically¹. Aortic strips with or without endothelium will both respond with contraction to noradrenaline or α -selective adrenergic agonists. However, if acetylcholine is applied subsequently, only the strip that retains its endothelium will respond with relaxation, whereas the one without will stay contracted. Thus, the endothelium is required for the relaxation of the smooth muscle to occur

¹ By limited proteolysis using collagenase – a protease that is also commonly used to disintegrate tissues into their constituent cells for the sake of obtaining pure cultures of individual cell types.

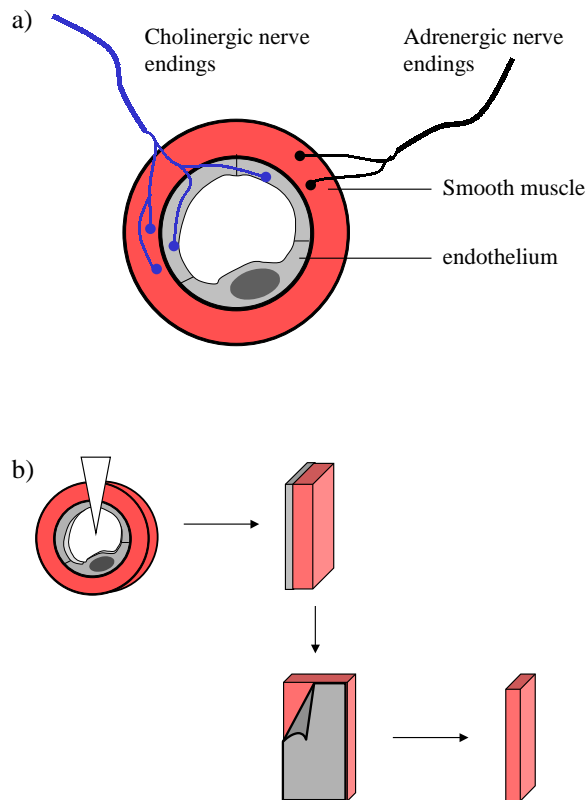


Figure 11.1. Innervation of vessel walls by cholinergic (parasympathetic) and adrenergic (sympathetic) nerve endings (a), and preparation of aortic strips from ring-shaped slices for experimental study of vascular smooth muscle contraction (b).

(Figure 11.2a). If a strip with the endothelium removed is placed next to one retaining the endothelium, and the entire assembly is exposed to first norepinephrine and then acetylcholine, the endothelium-less strip will still relax, indicating that EDRF is a diffusible substance (Figure 11.2b).

Approximately at the same time that EDRF was identified as NO, it was found that the common mode of action of several drugs containing nitrate or similar functional groups consisted in the release of nitric oxide. The identity of EDRF and NO was initially suspected based on circumstantial evidence: Both NO and EDRF were found to bind to and be inactivated by hemoglobin (Figure 11.3a). Furthermore, the effects of both were augmented by superoxide dismutase. This enzyme scavenges superoxide anions by disproportionation into O₂ and H₂O₂. Since superoxide reacts very rapidly with NO, superoxide dismutase will increase the lifetime and biological effect of NO (Figure 11.3b). The identity was finally established by the direct

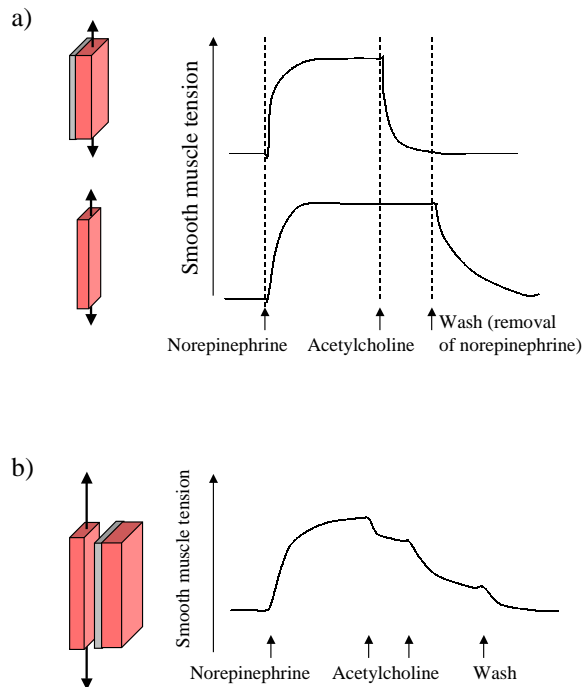


Figure 11.2. Contraction and relaxation of aortic strips (cf. Figure 11.1) in response to norepinephrine and acetylcholine. a: The endothelium is not needed for contraction but is necessary for relaxation. b: The relaxing factor contributed by the endothelium can act on a second, not anatomically connected strip of vessel wall devoid of epithelium, indicating that it is diffusible.

detection of NO in biological samples using a chemiluminescence assay (Figure 11.3c).

11.2. Nitric oxide synthase and its isoforms

NO is generated *in vivo* by nitric oxide synthase (NOS). This enzyme is located in the cytosol and utilizes arginine, molecular oxygen, and NADPH as substrates (Figure 11.4a). NOS is a fairly complex molecule that possesses multiple redox coenzymes which constitute a little electron transport chain of their own. NOS is a dimer, and the electron transfer actually occurs between the two subunits (Figure 11.4b). There are several subtypes of NOS: Endothelial NOS or eNOS, neuronal NOS or nNOS, and inducible NOS or iNOS. eNOS is responsible for the blood vessel-relaxing effect discussed above. Of note, it is found in both arteries and veins. Accordingly, its activation will both lower resistance (by arterial relaxation) and increase volume capacity (by venous relaxation), and therefore cause a strong reduction of blood pressure. In fact, NO-releasing drugs are the most powerful vasodilators available, overriding the action of other mediators such as norepinephrine (see above). They also have a very prompt onset of action, and they are used when lowering of blood pressure or release of vasospasms must be achieved immediately.

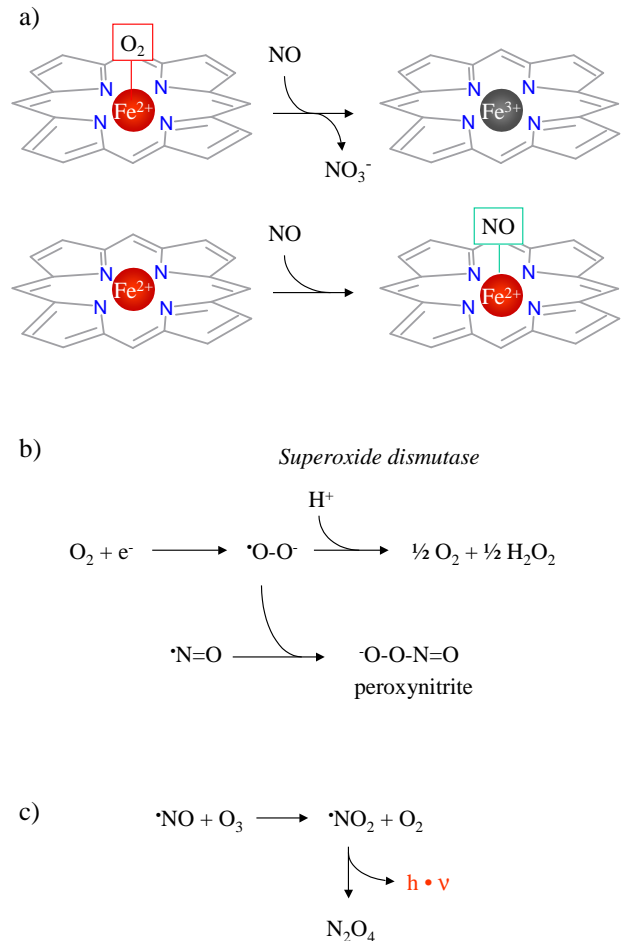


Figure 11.3. Experimental findings that led to the identification of endothelium-derived relaxing factor (EDRF) as NO. The following observations were made with both EDRF and NO: a: Inactivation by oxyhemoglobin, associated with formation of methemoglobin (top), and binding to hemoglobin. b: Extended lifetime in the presence of superoxide dismutase. c: Luminescence upon reaction with ozone.

Neuronal NOS is found in the central nervous system, where NO fulfills the role of yet another neurotransmitter. NO signalling between neurons works in much the same way as between endothelial and smooth muscle cells, i.e. it does not involve a receptor on the cell surface (see below). iNOS is found in macrophages, which are one of the major types of phagocytic cells. NO released within and from macrophages serves an entirely different purpose – that of an antimicrobial effector mechanism (see later). The two different roles of NO – mediator in blood vessels and the central nervous system (CNS), microbicide in the macrophage – are reflected by different control mechanisms of the corresponding NOS isoforms. Whereas eNOS and nNOS are controlled by Ca⁺⁺ / calmodulin (providing for very rapid and dynamic control), iNOS is controlled by transcriptional activation (which is much slower but also much longer sustained).

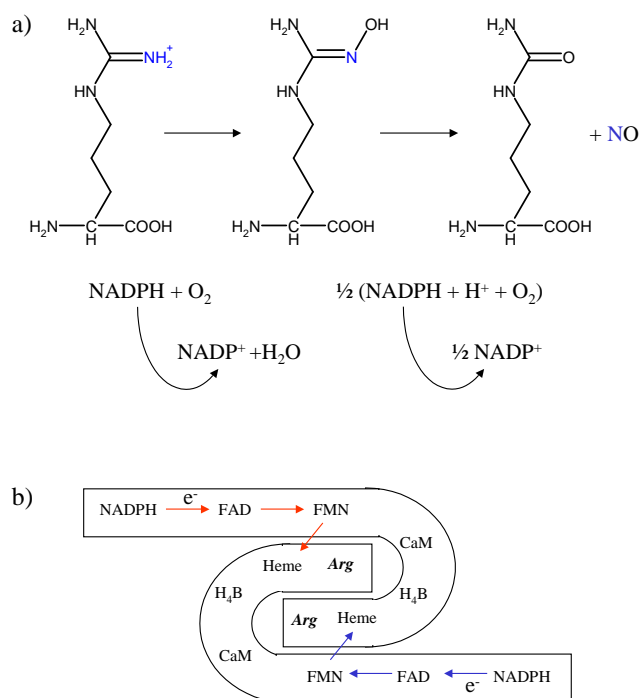


Figure 11.4. Reaction catalyzed by Nitric oxide synthase (a), and arrangement of the coenzymes in the dimer (b). Electrons flow from NADPH and flavins of one subunit to the heme of the other. Arg denotes the substrate binding site. Tetrahydrobiopterin (BH_4) also participates in electron transfer.

11.3. Biochemical mechanisms of NO signalling

How does NO signalling work? As mentioned above, NO is generated within the cytosol of the endothelial cell (or, in the CNS, the presynaptic cell). As it is able to cross cell membranes with ease, it will diffuse into neighbouring cells, i.e. the smooth muscle cells (in the blood vessel walls) or the post-synaptic nerve cells (in the case of nNOS). There, it will bind to a heme group that is attached to the enzyme called ‘soluble guanylate cyclase’ (sGC). NO binding will activate sGC, which will result in the synthesis of cyclic guanosine monophosphate (cGMP) from GTP, in a manner analogous to adenylate cyclase, which as we’ve seen forms cAMP from ATP. However, the molecular mechanism of sGC activation by NO is quite special: Binding of NO to one side of the heme moiety of sGC will break the bond of the heme iron to a histidine residue on the opposite side, which in turn triggers the conformational change that leads to activation (Figure 11.5a). The heme moiety seems to be there solely for the purpose of NO binding but not to be part of the active site.

cGMP is a second messenger with a variety of effector mechanisms (Figure 11.5b), the foremost one of which is the activation of a cognate protein kinase², common-

²Actually there are several such kinases; the name ‘PKG’ is collectively applied to them.

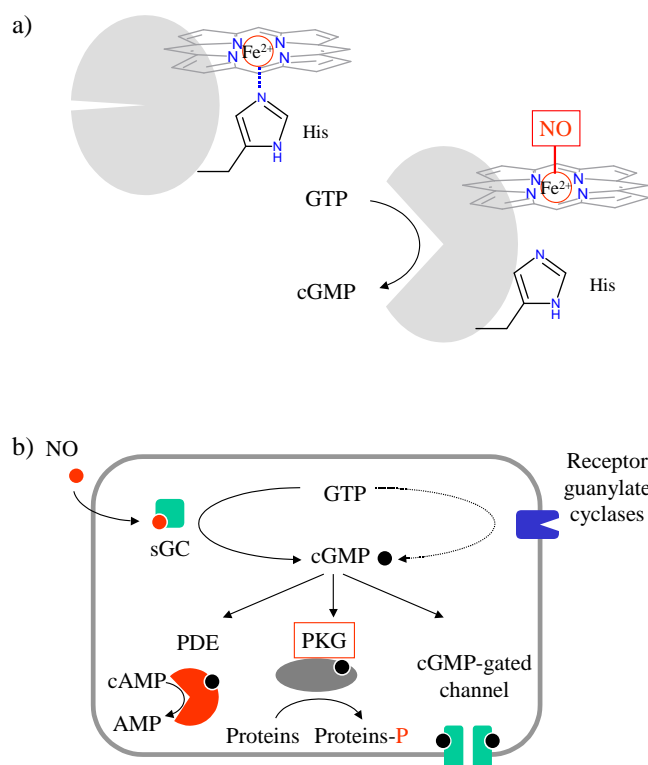


Figure 11.5. Mechanism of signalling by NO. a: NO binds to a heme group of soluble guanylate cyclase (sGC). This causes allosteric activation of the enzyme and results in increased cGMP levels. b: cGMP controls several types of target proteins: cAMP-selective phosphodiesterase is activated, so that cAMP levels will be reduced. Protein kinase G (of which there are several types) will be activated; this appears to be the most important single mechanism. In some cells, there are cGMP-gated channels that will close upon binding of cGMP. (Receptor guanylate cyclases provide an alternate means of cGMP production that is independent of sGC and NO.)

ly referred to as ‘protein kinase G’ (PKG; G for cGMP-dependent). In addition, cGMP also controls ligand-gated ion channels in sensory cells as well as in nerve cells and smooth muscle cells; not much is known on the contribution of such channels to NO signalling. Finally, cGMP activates phosphodiesterase, which will inactivate cAMP by cleavage to AMP. Thus, cGMP is somewhat of an antagonist of cAMP.

How does cGMP bring about relaxation of vascular smooth muscle? Several mechanisms, all of which involve PKG, have been proposed; none of them is at present certain to be ‘the’ major one. Moreover, while several relevant changes in protein phosphorylation have been observed, it is not clear at present whether they are caused by PKG directly or by intervening secondary kinases.

In smooth muscle, contraction is controlled by the phosphorylation state of the myosin regulatory light chain. The extent of this phosphorylation will depend on the regulatory states of both myosin light chain kinase (MLCK) and of

myosin light chain phosphatase. It seems that PKG phosphorylates the phosphatase, thereby increasing its activity (Figure 11.6a).

Another plausible candidate effector molecule for cGMP / PKG is the IP_3 receptor channel in the endoplasmic reticulum (Figure 11.6b). Its phosphorylation reduces release of Ca^{++} from the ER in response to, e.g., adrenergic stimuli (recall that α_1 -receptors signal through IP_3 / Ca^{++}). The two mechanisms combined would cause both decreased phosphorylation and increased dephosphorylation of myosin light chains. There is also experimental evidence indicating that phospholipase C is inhibited by PKG, which would lead to inhibited formation of IP_3 , and that Ca^{++} -ATPases in both the ER and the cytoplasmic membrane may be activated. Since Ca^{++} -ATPases remove Ca^{++} from the cytosol, this would further reduce the availability of Ca^{++} for contraction. While all these mechanisms appear plausible, it is impossible at present to assess their relative importance in the effect of cGMP- and NO-mediated signalling. However, given the very strong relaxing effect of NO on the vas-

cular smooth muscle, it is reasonable to assume that more than one mechanism contributes significantly.

The second aspect of NO that needs to be considered is its chemical reactivity. In fact, NO binds similarly well to the heme moieties in hemoglobin and in guanylate cyclase, and reaction with hemoglobin is often considered a major mechanism of inactivation of NO. However, NO bound to non-oxygenated heme may be released again, so that hemoglobin may actually serve as a carrier. Alternatively, NO may be transferred from heme to protein cysteine sulfhydryl groups. One quite reactive such cysteine residue is located within hemoglobin itself. By removing NO from heme, the cysteine becomes S-nitrosylated itself.

Heme does, however, not seem to be required for protein S-nitrosylation to occur. The precise chemistry is unsettled; Figure 11.7a gives one hypothetical reaction scheme. Here, S-nitrosylation generates one equivalent of superoxide, which in turn (and particularly so at high concentrations of NO) may react with another molecule of NO to generate peroxynitrite. Peroxynitrite is very reactive and may oxidize other sites in proteins, or it may give rise to O-nitrosylation of protein tyrosine side chains.

While different reaction mechanisms for S-nitrosylation have been proposed, a common motif in all of those I have seen is the participation of oxygen, which makes sense, as the hydrogen of the sulfhydryl group must be disposed of. NO groups can also be transferred from one sulfhydryl group to another (Figure 11.7b), so that covalently bound NO is rarely ever excluded from further circulation. This also means that protein S-nitrosylation should be reversible by way of transferring the nitrosyl group to a low-molecular weight thiol compound such as, e.g., free cysteine or glutathione.

That S-nitrosylation indeed occurs *in vivo* is illustrated in Figure 11.8. In the experiment depicted, the S-nitrosylated proteins have been visualized using an antibody that specifi-

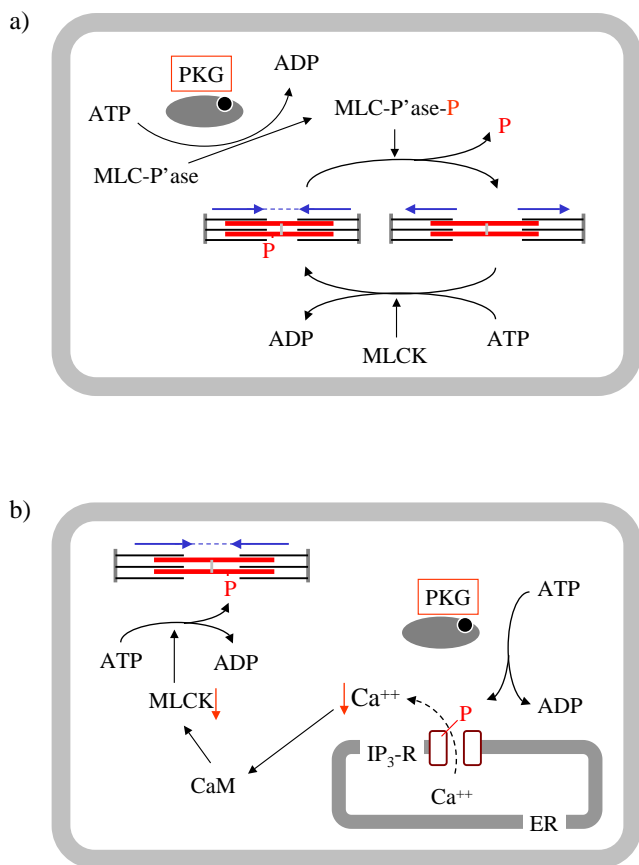


Figure 11.6. Mechanisms of smooth muscle relaxation induced by cyclic GMP and protein kinase G (PKG). a: PKG phosphorylates and thereby activates myosin light chain phosphatase. b: PKG also phosphorylates the IP_3 receptor, which results in reduced efflux of Ca^{++} from the endoplasmic reticulum. Ca^{++} is involved in smooth muscle cell contraction via calmodulin and myosin light chain kinase.

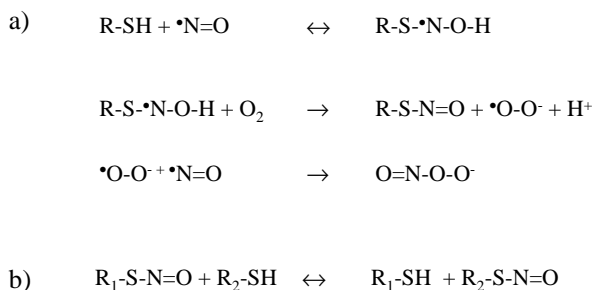


Figure 11.7. Reactions of nitric oxide involving sulfhydryl groups. a: Hypothetical mechanism of nitrosothiol formation. The superoxide anion generated may react with a second molecule of NO to yield peroxynitrite. b: 'Migration' of nitroso groups between sulfhydryls. This is experimentally illustrated in Figure 11.9b.

cally recognizes the -S-N=O group as its epitope. This antibody is targeted with a secondary antibody, which in turn is coupled to an enzyme. The latter releases a coloured, insoluble product from a soluble 'chromogenic' precursor (Figure 11.8a). Localized precipitation of the insoluble stain will thus highlight the distribution and extent of protein S-nitrosylation.

As expected, the most intensely stained region is the endothelium itself (Figure 11.8b, middle panel); this makes sense, as the concentration of NO should be highest at the site of formation. However, the surrounding smooth muscle tissue is stained as well, suggesting that indeed S-nitrosylation might contribute to the muscle relaxation triggered by NO. That nitrosylation is indeed due to the activity of NOS, and that detection is reliable is evident from the fact that in the presence of an inhibitor of NOS no stain is accumulated (Figure 11.8b, right panel).

Does S-nitrosylation really have a regulatory role in the cell? This raises the question how specific protein S-nitrosylation might be. From the non-enzymatic nature of the chemistry discussed above, one might expect S-nitrosylation to be a very indiscriminate process, which would amount to a lot of 'noise' in the signal – and thus, probably,

no real signal at all, just noise. The number and identity of proteins affected by S-nitrosylation has been studied using a rather ingenious experimental approach, outlined in Figures 11.9 and 11.10. In these experiments, the nitrosylated sulfhydryl groups were derivatized with biotin, which facilitates selective detection and / or purification³. The challenge here consists in avoiding those cysteine residues that are either free or part of disulfide bonds. The free ones were first blocked with the reagent methylmethanethiol-sulfonate (MMTS). While one way to reduce nitrosothiol groups would be reaction with an excess of low molecular weight thiol (such as dithiothreitol or 2-mercaptoethanol), this would also reduce the disulfide bonds (both the cysteines and the newly formed MMTS derivatives). Selective reduction of nitrosothiols only can be achieved using ascorbic acid; in this way, only the formerly nitrosylated cysteines will be amenable to biotinylation. Biotinylated proteins can be selectively detected, after gel electrophoresis and blotting, with streptavidin that is coupled to an enzyme, again using a chromogenic substrate (Figure 11.9).

Figure 11.9b shows that a spectrum of proteins is affected by S-nitrosylation within a sample from neuronal cells. The nitrosylating agent, in this case, was not NO itself but S-nitroso-glutathione, illustrating the fact that indeed the NO moiety may travel easily between sulfhydryl groups (cf Figure 11.7b). Given the fact that most proteins should have one or more free cysteine residues, the number of proteins that are detectably labeled is surprisingly small. Stained bands were recovered from the blots and identified by 'proteomics' methods, i.e. proteolytic fragmentation, mass spectrometry, and Edman degradation. Some of the names of identified proteins seem to 'ring a bell' with respect to possible involvement in signal transduction cascades – e.g., ion channels (including the NMDA subtype glutamate receptor); others, however, don't (e.g. tubulin, actin⁴).

The same authors also examined the S-nitrosylation of proteins by nNOS, in neuronal tissue from mice (Figure 11.10). Conversion of nitrosylated to biotinylated cysteines was done as above. Biotinylation was, in this case, used to extract the (formerly) nitrosylated proteins from the total mixture of cellular proteins by selective binding to solid-phase attached streptavidin. After retrieving the bound material by reduction with excess free thiol, the samples were run on a gel, blotted, and individual proteins detected using an

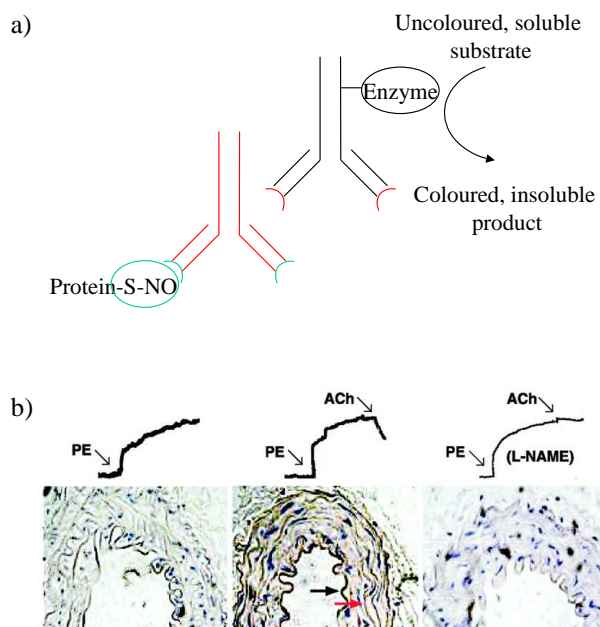


Figure 11.8. Detection of NOS-dependent S-nitrosylation *in vivo*. a: Immunochemical detection procedure. A primary antibody directed against nitrosothiol groups was used in combination with an enzyme-linked secondary antibody. The dye released by the enzyme is insoluble and thus will precipitate close to the site of formation. b: S-nitrosylation in aortic rings. Samples were treated with Phenylephrine (PE), acetylcholine (ACh), and the inhibitor N-methylarginine (L-NAME) as indicated. Brown stain deposits indicate S-nitrosylation. The traces above the histology panels illustrate the contraction and relaxation responses evoked by the drugs.

³Why did the authors not use the anti -S-N=O antibody instead? Maybe they didn't think of it. However, I suspect that the antibody might not possess sufficient affinity to allow for stringent washing of blots and columns. The biotin-streptavidin interaction is very stable and thus withstands the stringent washing steps required for selective detection within complex protein mixtures such as whole cell extracts.

⁴Actin might still be interesting in the context of smooth muscle relaxation, though.

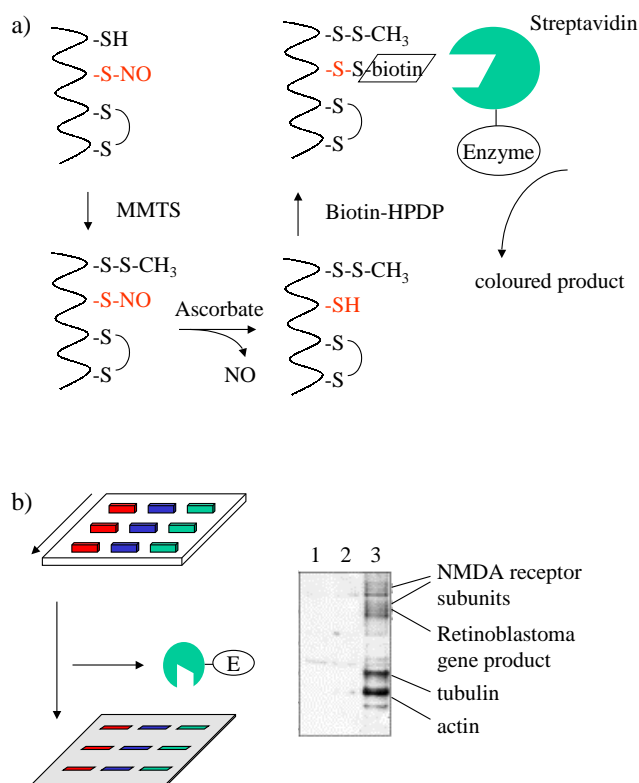


Figure 11.9. Identification of proteins amenable to S-nitrosylation. a: Selective biotinylation of S-nitrosylated proteins. Remaining free thiols were blocked first with methylmethanethiolsulfonate (MMTS). Selective reduction of nitrosothiols was achieved with ascorbic acid; this was followed by biotinylation. Biotin is useful for selective detection with enzyme-coupled streptavidin. b: Analytical separation of S-nitrosylated (now biotinylated) proteins. Cell extracts were treated with the S-nitrosylating agent S-nitrosoglutathione, resolved by gel electrophoresis, blotted, and the blots developed with streptavidin-peroxidase. Lane 1: No reducing or nitrosylating agent was present; lane 2: Glutathione 40 μ M, lane 3: S-nitrosoglutathione (40 μ M). Proteins were identified after excision from the gel; some selected names are given. *Data reproduced with permission from Nat Cell Biol. 3:193-7 (2001)*

tibodies directed not against S-N=O (it's no longer there, is it) but against those proteins themselves. Figure 11.10b shows a roundup of several nitrosylated proteins. For comparison, the starting material (the total protein extract, without pre-selection by streptavidin binding) was run next to the samples retrieved from the streptavidin column. Furthermore, dependency of nitrosylation on NOS activity was confirmed by parallel processing of samples from nNOS knockout mice (nNOS $-/-$).

What does all this tell us?

It has been claimed that S-nitrosylation may have a similarly fundamental regulatory role as protein phosphorylation has. Is this claim substantiated by the findings presented? My personal impression is that it is not. However, this area is presently under intense investigation, and protein nitro-

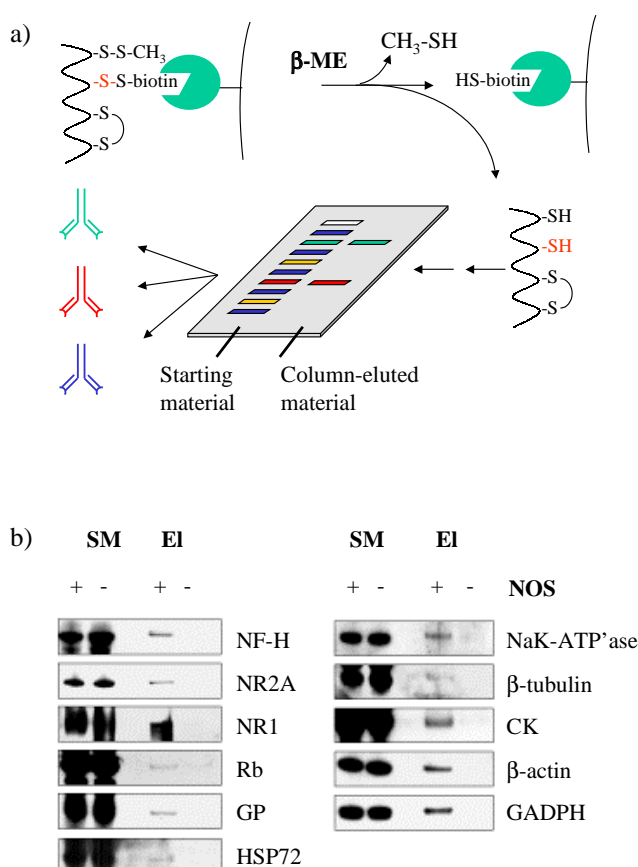


Figure 11.10. Identification of proteins subject to S-nitrosylation due to nNOS activity. a: Experimental strategy. Protein samples were selectively biotinylated as before (cf. Figure 11.9), enriched by adsorption to solid phase-bound streptavidin, blotted, and detected by specific antibodies. b: Results for several proteins. SM: Starting material (not passed through column; both S-nitrosylated and unmodified protein molecules will show up here). EI: Column eluate - this will only detect the nitrosylated proteins. +: Samples from wild-type mice (nNOS $+/+$), -: samples from knockout mice (nNOS $-/-$). *Data reproduced with permission from Nat Cell Biol. 3:193-7 (2001)*

sylation is considered by many as a viable mechanism of signal transduction.

11.4. Role of NO in macrophages

S- and O-nitrosylation are, however, very likely important in the second function of NO – i.e., in killing microbes by macrophages (Figure 11.11). Macrophages are the most potent phagocytic cells in the immune system, in charge of dealing with hardy microbes such as mycobacteria, which are completely resistant to other phagocytic cells such as granulocytes. In these cells, the NO concentrations are substantially higher than in nerve or endothelial cells. While reactive oxygen species have a substantial bactericidal effect in the absence⁵ of NO, the latter enhances the ability

of macrophages to kill bacteria. This may partially be due again to the ability of NO to cross membranes with ease, which would let it penetrate the interior of the microbial cell; most other effector molecules (including, e.g., the very toxic superoxide anion) cannot do this. Inside the microbial cell NO might again act by binding to heme, e.g. within the microbial respiratory chain. In addition, NO also reacts with molecular oxygen or reactive oxygen species to yield peroxynitrite or N_2O_3 , which will broaden the spectrum of antibacterial reactivity within and without the macrophage⁶ or microbes.

Why does inflammatory NO release matter here? In states of severe infection, patients may develop a state of pathologically low blood pressure, known as *septic shock*⁷, which frequently is the ultimate cause of death. One of the mechanisms that are considered to be responsible is the massive release of NO from macrophages. Accordingly, one of the therapeutic strategies that are being tried in septic shock consists in the application of NOS inhibitors (see below).

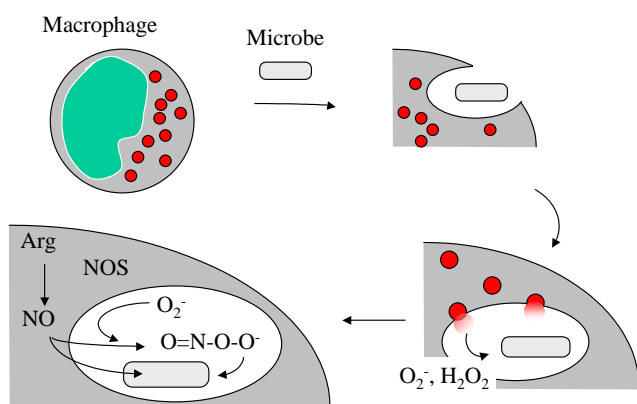


Figure 11.11. Role of NO in the killing of microbes by macrophages. Microbes are ingested, and peroxisomes fuse to the phagosomes, releasing reactive oxygen species. NO enters the phagosomes and either forms peroxynitrite by reaction with reactive oxygen species, or it diffuses across the microbial cell wall to wreak havoc inside.

⁵ H_2O_2 reacts, for example, with chloride to form HOCl (hypochloric acid), which is known as bleach and renowned for antibacterial activity. Formation of HOCl *in vivo* is catalyzed by myeloperoxidase. Its importance is underscored by the fact that people lacking that enzyme suffer from a quite pronounced immune deficiency, in particular with respect to bacterial infections.

⁶ Macrophages act against both ingested microorganisms, as illustrated here, and extracellular microbes.

⁷ Shock, as a medical term, has nothing to do with ‘embarrassment’, ‘terror’ or Donald (‘shock’n awe’) Rumsfeld. Instead, it refers to a state of dangerously low blood pressure, in which minimal perfusion of some organs is no longer guaranteed. This may be caused by sudden loss of blood (hypovolemic shock), heart failure (cardiogenic shock), or vasodilatation (most commonly septic shock, although it can also be induced by drugs).

11.5. NO releasing drugs

So, what does a NO releasing drug look like? The first one there was (Figure 11.12, top) is more widely known in another application, which helped Mr. Alfred Nobel make his fortune – and still maintain his good conscience, since he believed that this weapon would be so horrible that mankind would henceforth abstain from warfare (a hope now held by many for nuclear weapons). The effect of nitroglycerine was initially noted in the form of the ‘Monday headache’: The factory workers, returning to work on Monday, experienced a strong headache⁸ that faded away with continuous exposure to nitroglycerine vapours during the workweek, only to reappear the next Monday after withdrawal during the weekend. Nitroglycerin – applied sublingually as a spray for rapid uptake and avoidance of liver first-pass effect – is still the standard treatment of acute events of angina pectoris, which is basically an acute, painful deterioration of coronary artery perfusion, caused by vasoconstriction on top of atherosclerotic lesions. One of the first patients benefiting from this treatment was – Nobel himself. I don’t know whether he actually troubled himself to get it from the pharmacy, though.

Isosorbide dinitrate, like nitroglycerin, contains NO within nitrate groups (Figure 11.12, bottom). Amylnitrite (which is volatile and can be inhaled) has a nitrite instead of the nitrate group. Yet another chemistry is found with sodium nitroprusside. While release of NO from all these drugs *in vivo* is rather fast (particularly so with nitroprusside), the mechanisms are apparently different. However, until today there is no clear picture exactly how this works. Reaction with thiol compounds *in vitro* will release NO but (at least with nitrites and nitrates) is too slow to account for the almost instantaneous onset of drug action *in vivo*. One publication in 1993⁹ described a protein (referred to by the authors as an enzyme, but no physiological function was giv-

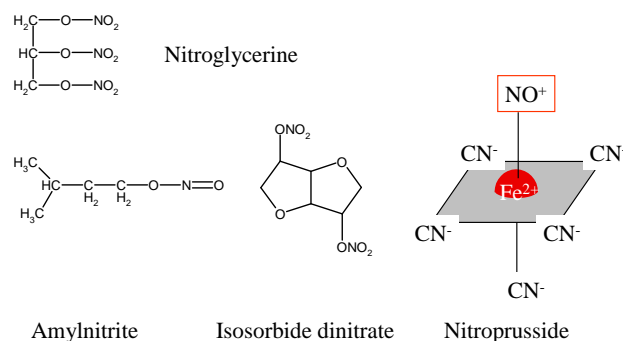


Figure 11.12. Structures of nitric oxide-releasing drugs.

⁸ Headache is commonly linked to disturbed vasomotor function, and some of the drugs used in migraine (such as ergotamine) are in fact vasoconstrictors.

⁹ Biochem Pharmacol. 46:1481-6 (1993)

en) that accelerated NO release from nitrates and was partially purified from smooth muscle cell membranes. It was inhibited by thiol reagents, suggesting the occurrence of a cysteine in the active site. While this demonstrates the possibility of protein-catalyzed release, it is unclear whether this particular protein has a key role in it *in vivo*. Although this appears interesting, I have not found any study following up on it. Knowledge about the mode of NO release from nitrites is similarly scanty. Nitroprusside releases NO faster with low-molecular weight thiols than nitrates do, suggesting that enzymatic catalysis may not be necessary *in vivo* in this case.

11.6. NOS inhibitors

Inhibitors of NOS, while widely used in experimental research, are still in under investigation for clinical application. As mentioned above, the release of endogenous NO may be pathologically enhanced in septicæmia, leading to septic shock. Treatment with NOS inhibitors has been suggested as early as ten years ago but is still in the experimental stage. Inhibitors of iNOS are also of interest (and at the

stage of animal experiments) in the treatment of chronic inflammatory diseases, e.g. rheumatoid arthritis. Some such drugs are derivatives of arginine (Figure 11.13a). Interestingly, very simple alkyl derivatives of isothiourea are very potent inhibitors of NOS (Figure 11.13b). For clinical use it would, of course, be very favourable to have isoform-selective inhibitors. Some experimental inhibitors that indeed do show some preference for iNOS and nNOS, respectively, are shown in Figure 11.13c. In particular, selective inhibition of iNOS should be advantageous in septic shock and in chronic inflammatory diseases. Data from animal models of septicaemia and of arthritis are promising.

Finally, a discussion of nitric oxide would not be complete without mentioning one of the most successful drugs of the past decade – sildenafil¹⁰, more widely known under its trade name (Viagra). Sildenafil is an inhibitor of the phosphodiesterase subtype 5, which is selective for cGMP (Figure 11.14). This enzyme removes cGMP and thus terminates the action of NO (if protein nitrosylation is neglected). Sildenafil was not developed with its now-famous application in mind; instead, the idea was to come up with another vasodilator. Its enhancing effect on penile erection gave rise to the discovery that NO actually is the transmitter that triggers this process. Thus, without NO, no one of us would even be here today!

In theory, it should be possible to further augment the effect of sildenafil with nitrate drugs, shouldn't it? However, this cannot be recommended¹¹ – it has been tried and resulted in heart attacks. Sildenafil may also cause disturbances of colour vision; this is due to the role of cGMP-gated channels in the excitation of the sensory cells of the retina.

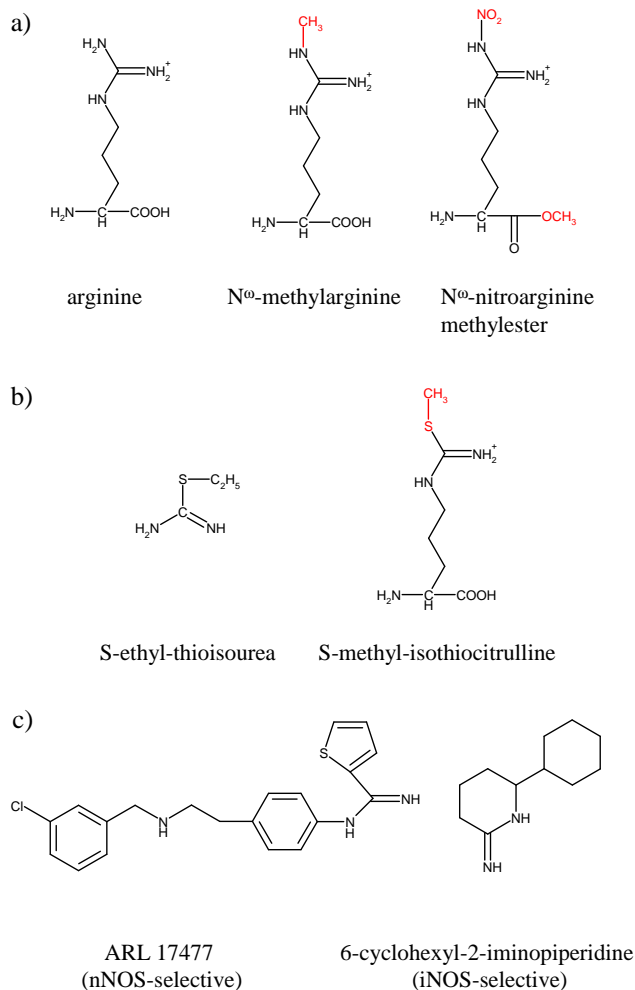


Figure 11.13. Inhibitors of nitric oxide synthase.

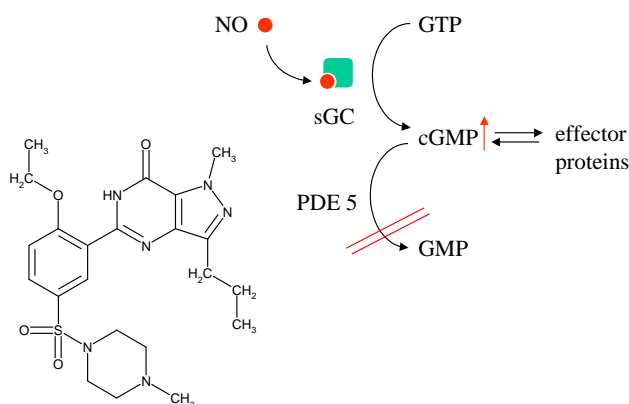


Figure 11.14. Structure of sildenafil (Viagra®), and sketch of its mechanism of action: Inhibition of phosphodiesterase increases cGMP levels.

¹⁰ Just how successful I noticed when typing this – the spell checker didn't object, which it did even with nitroglycerin.

¹¹ Except, maybe, for obnoxious fathers-in-law – which recommendation is politically correct inasmuch it restores the balance to the recipe previously given for mothers-in-law.

(Notes)

Chapter 12. Pharmacology of Eicosanoids

Eicosanoid mediators are derived from arachidonic acid (eicosatetraenoic) and related poly-unsaturated fatty acids, such as acid eicosapentanoic acid. These fatty acids are mainly found as constituents of phospholipids in cellular membranes (Figure 12.1a), and it is from there that they are mobilized for eicosanoid mediator synthesis. The major classes of eicosanoids are prostaglandins, thromboxanes, and leukotrienes. Eicosanoids are very widespread in the mammalian organism – most cells synthesize them.

Eicosanoids are involved in normal physiological processes such as:

- Regulation of blood flow and urine processing in the kidney: Prostaglandin E (PGE), PGF, PGI (also called prostacyclin), and thromboxanes (TXA)
- Bone metabolism (deposition and mobilization of calcium¹: PGE and PGF
- Integrity of gastric mucosa: PGE and PGF
- Uterine contraction (labour): PGE and PGF
- Gastrointestinal motility: PGE, PGF, PGI
- Thrombocyte aggregation and activation (stimulated by TXA, inhibited by PGE and PGI)

As you can see, the individual eicosanoid mediators typically have multiple roles; from this, we can already infer that drugs acting on the eicosanoid metabolism will be prone to side effects.

Eicosanoids are also involved in disease-related phenomena:

- Inflammation: PGE, leukotrienes (LTC₄, LTD₄)
- Fever (PGE)
- Pain (PGE)

Eicosanoid receptors are G protein-coupled receptors; known effector mechanisms include activation or inhibition of adenylate cyclase and activation of phospholipase C. Duration of action is usually short; inactivation occurs by oxidative metabolism either locally or in the lung, which is strategically placed for complete clearance of the circulating blood volume.

12.1. Biosynthesis of eicosanoids

The first step in the formation of eicosanoid mediators consists in the release of the precursor fatty acids from the membrane phospholipids. This release may happen along several possible metabolic routes (Figure 12.2a). The major physiological mechanism of release consists in the activation of a cytosolic phospholipase A₂ (cPLA₂) by Ca⁺⁺ in response to an extracellular signal. cPLA₂ then attaches to the nuclear (and probably ER) membranes, which appear to be the major reservoir of arachidonic acid and its analogs.

¹Calcium turnover in the bone is a highly dynamic process; the bone serves as a reservoir for circulating and intracellular calcium.

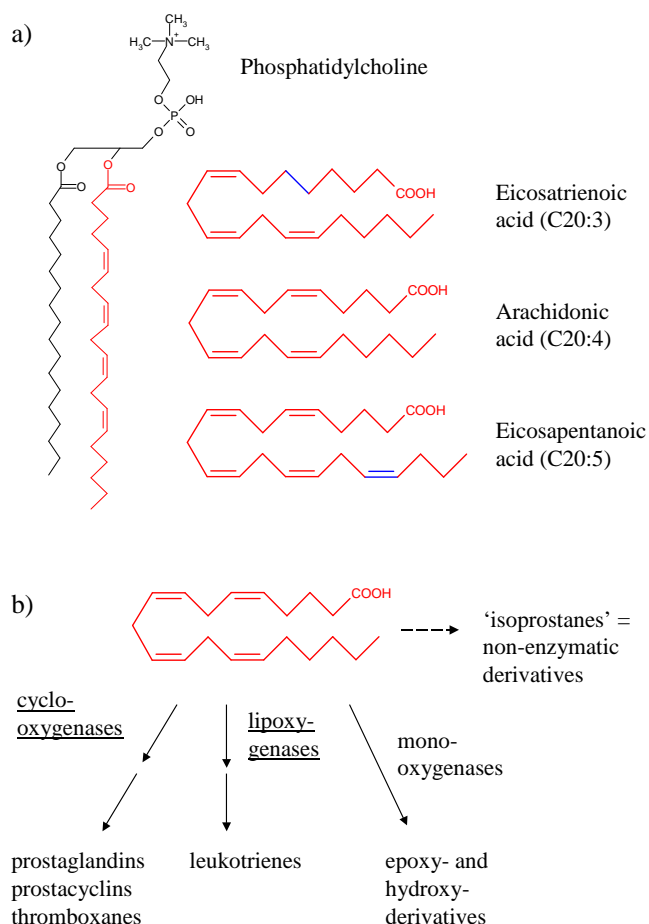


Figure 12.1. Overview of eicosanoid metabolism. a: Structures of eicosanoid precursor fatty acids, and their occurrence in membrane phospholipids (PC is shown as an example). Arachidonic acid is the 'prototypic' precursor; eicosatrienoic and eicosapentanoic acid differ from it by the lacking or an additional double bond, respectively. b: Conversion of precursor fatty acids occurs by various enzymes, notably cyclooxygenases and lipoxygenases. 'Isoprostanes' are non-enzymatic derivatives that may form *in vivo* at appreciable rates; one characteristic feature is that they occur as racemic mixtures. Their physiological significance is not entirely clear.

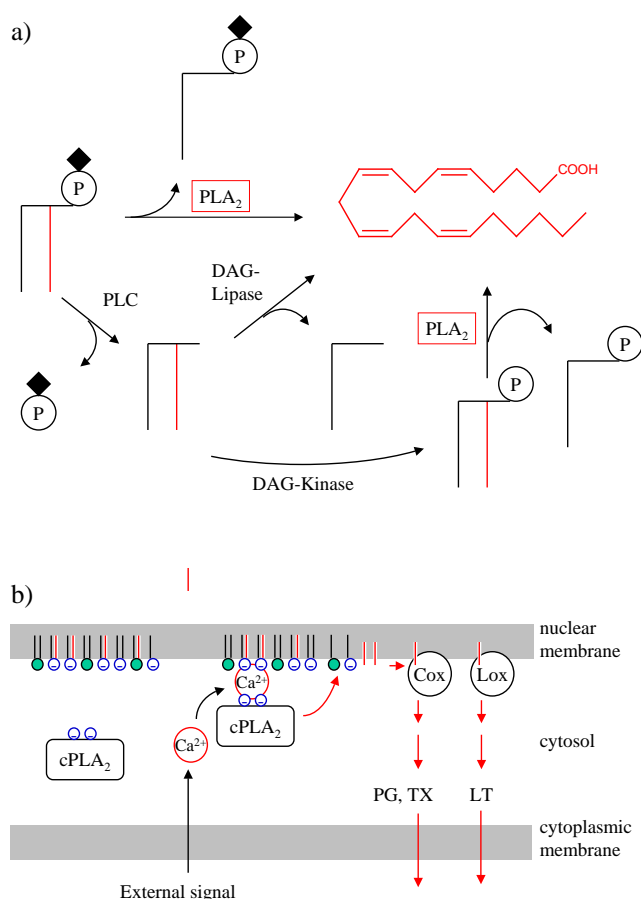


Figure 12.2. Alternate pathways of arachidonic acid release (a), and cellular locations of enzymes involved in eicosanoid formation (b). a: Arachidonic acid may be directly released by phospholipase A₂ (PLA₂), or alternatively by the successive action of phospholipase C (PLC) and diacylglycerol (DAG) lipase. b: The major mechanism of release involves a cytosolic phospholipase A₂ (cPLA₂). An increase of Ca²⁺ in response to an extrinsic signal causes binding of cPLA₂ to the nuclear membrane. Cyclooxygenase (COX) and Lipoxygenase (LOX) form their respective intermediates, which are further processed by cytosolic enzymes to prostaglandins (PG), thromboxanes (TG), and leukotrienes (LT), respectively.

The formation of the most important eicosanoid derivatives of arachidonic acid and its analogs is initiated by cyclooxygenases (Cox) and lipoxygenases (Lox):

- Cyclooxygenase, also called prostaglandin H synthase, converts arachidonic acid first into prostaglandin G₂ (PGG₂) and then PGH₂. PGH₂ is the common precursor of prostaglandins E₂ and F₂, and of prostaglandin I₂ (prostacyclin). It is also the precursor of thromboxane A₂ (TXA₂). Therefore, cyclooxygenase is the single most important enzyme and drug target in eicosanoid metabolism.
- Lipoxygenase 5 converts arachidonic acid into 5-hydroperoxy-eicosatetraenoic acid (5-HPETE), which is the precursor of leukotrienes. Leukotrienes

are formed mainly in leukocytes, e.g. macrophages and granulocytes, and they are potent pro-inflammatory mediators. Suppression of leukotriene synthesis with inhibitors of lipoxygenases is a fairly recent therapeutic principle in the treatment of asthma and chronic inflammation.

- 12- and 15-HPETE are formed by the corresponding lipoxygenases 12 and 15. They can be reduced to hydroxy-eicosatetraenoic acids (HETEs) and further converted to lipoxins (cf Figure 12.12). H have their own receptors and physiological roles, but they are not presently in the focus of interest of drug therapy or development.

The major ‘classical’ drug target in prostaglandin metabolism is cyclooxygenase, which occurs in several isoforms: Cox-1, Cox-2, and apparently in some mammals Cox-3. This is due to the central role of its product prostaglandin H₂ as a precursor of multiple eicosanoid mediators (Figure 12.4).

The synthesis of PGH₂ occurs in two separate successive reactions (Figure 12.3), for which there are two separate active sites on the cyclooxygenase molecule². The first reaction – also referred to as the cyclooxygenase reaction – introduces two peroxy groups, one endoperoxide (forming a ring with carbons 9-11) and a hydroperoxide attached to po-

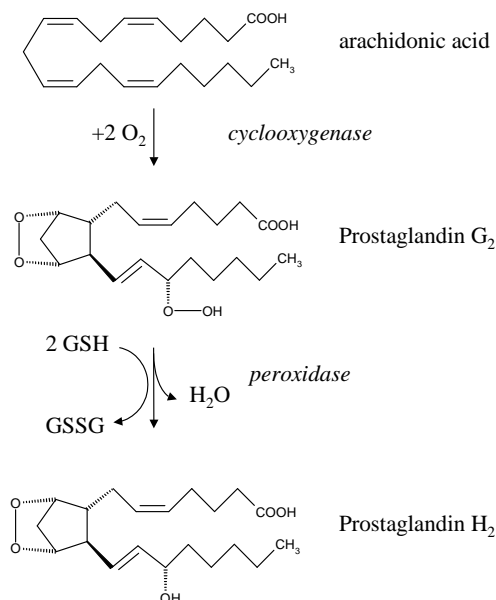


Figure 12.3. The two reactions catalyzed by cyclooxygenase. For each of these reactions, there is a separate active site (cf. Figure 12.5).

²The numerical indices in the names of prostaglandins and leukotrienes denote the number of carbon-carbon double bonds, which varies depending on the particular precursor fatty acid. The derivatives mentioned in the text (such as PGH₂ and LTD₄) are the derivatives of arachidonic acid, unless otherwise stated.

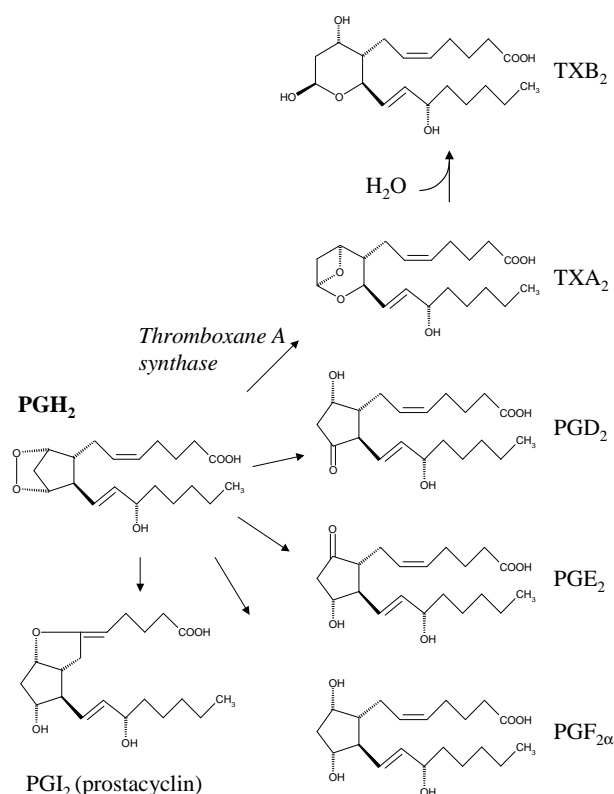


Figure 12.4. Structures of prostaglandin H_2 and its derivatives. PG, prostaglandin; TX, thromboxane.

sition 15. The resulting product—prostaglandin G_2 —is quite unstable, yet it is able to dissociate from the enzyme and to rebound to the second active site of the same or another enzyme molecule. There, the hydroperoxide is reduced to a simple hydroxy group, at the expense of two equivalents of reduced glutathione. This second step is called the peroxidase reaction.

The two active sites of cyclooxygenase are located close to each other (Figure 12.5a), and it is believed that this proximity is important in the ‘priming’ of the cyclooxygenase site. The first step in the cyclooxygenase reaction (Figure 12.6) is initiated by a tyrosyl radical (Tyr385 in cyclooxygenase 1³; Figure 12.5b,c).

This tyrosyl radical will not exist in a newly translated enzyme molecule, and once it is there, it may be lost due to capture of a hydrogen from somewhere else than the substrate. There thus has to be a mechanism for its formation or regeneration. This mechanism is provided by the heme in the peroxidase active site. The heme radical cation, which forms as an intermediate during the peroxidase reaction, can abstract a hydrogen atom from the tyrosine -OH group, which thus may act as a reductant in place of one of

³ The amino acid position numberings of the active site residues are somewhat different in cyclooxygenase 2 and 3.

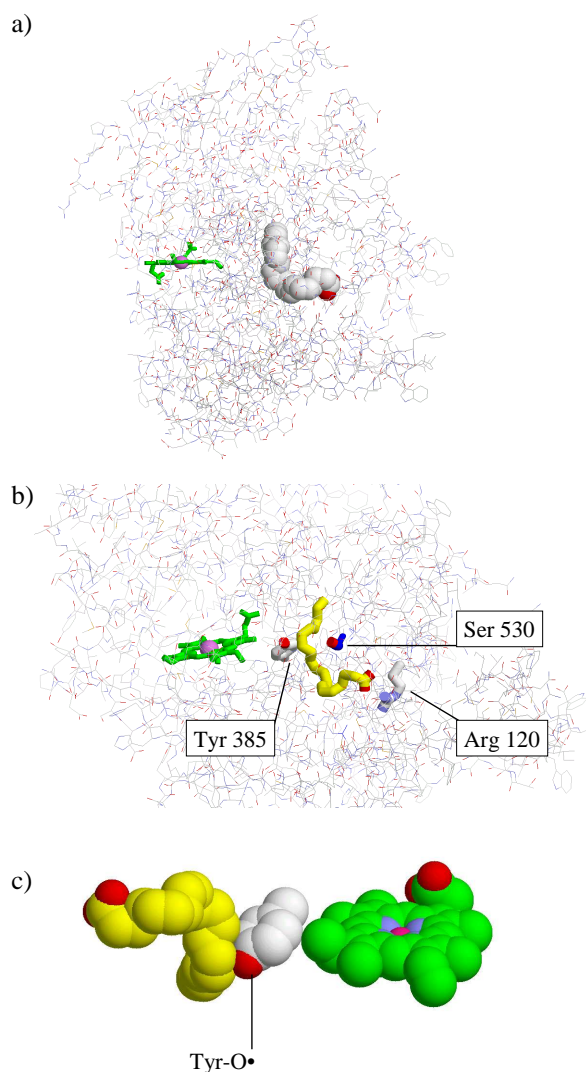


Figure 12.5. Spatial relationship of the two active sites in cyclooxygenase 1. a: Heme (green, with iron in magenta) and arachidonic acid (white, space-filling) bound within the peroxidase and the cyclooxygenase sites, respectively. b: Location of three important amino acid residues within the cyclooxygenase active site. Note that Tyr 385 is positioned between the two active sites. c: Space-filling representation of arachidonic acid (yellow), Tyr 385 (white), and heme (green).

the glutathione molecules normally functioning as cosubstrates (Figure 12.7b).

The peroxidase reaction (Figure 12.7a) can function in the absence of cyclooxygenase activity, because the intermediate product (prostaglandin G_2) can be provided by another enzyme molecule. In accord with this model, a sample of cyclooxygenase, when expressed recombinantly and in the absence of arachidonic acid substrate, will initially be inactive, but it will exhibit ‘burst’ kinetics upon first contact with arachidonic acid, due to the cascading activation of more and more enzyme molecules by PGG_2 ⁴.

⁴ Which still does not account for the activation of the first enzyme

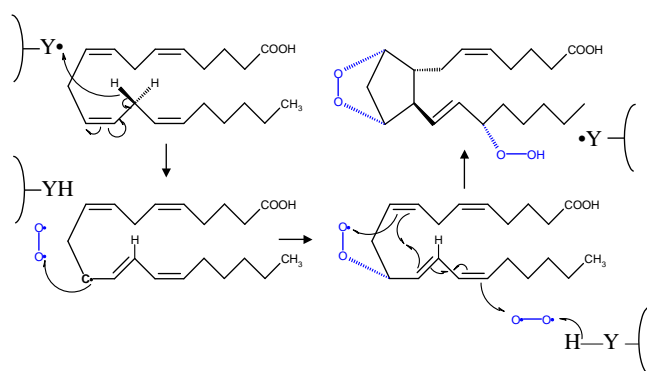


Figure 12.6. Catalytic mechanism of the cyclooxygenase reaction, leading from arachidonic acid (top left) to prostaglandin G_2 (top right). Y• and YH represent Tyr 385. Molecular oxygen reacts in its π -radical form. Note that this is only the first one of the two reactions catalyzed by cyclooxygenase.

12.2. Cyclooxygenase inhibitors

Cyclooxygenase occurs in three isoforms in the mammalian organism:

- Cox-1 is constitutively expressed and responsible for most of the ‘housekeeping’ functions of eicosanoids, including processes such as calcium metabolism in the bone, and stomach mucous membrane maintenance. It is also responsible for synthesis of thromboxanes in thrombocytes and of prostacyclin (PGI) in endothelial cells, which have antagonistic function in thrombocyte aggregation and activation (see later).
- Cox-2 is inducible and mostly expressed in inflammatory cells; it is considered the main culprit in the release of prostaglandins at sites of inflammation. Since anti-inflammatory therapy is the main therapeutic application of Cox inhibitors, there is considerable interest in the development of drugs selectively acting on this form.
- Cox-3 is a splice variant of Cox-1. It has been characterized in the brain of dogs. Its selective inhibition by acetaminophen, along with the antipyretic and analgesic⁵ activity of that drug, suggests a major role of Cox-3 in triggering fever and pain. However, if the homologous splice sites were used with a primary transcript of the human Cox-1 gene, this would give rise to an mRNA containing a premature stop codon. It is not clear at present whether humans actually do possess a third variant of Cox at all.

Most of the drugs that inhibit cyclooxygenase do inhibit both Cox-1 and Cox-2; this applies to drugs such as diclofenac, indomethacin, and acetylsalicylic acid. More recent developments have led to selective inhibitors of Cox-

molecule – some other oxidant has to be blamed, may be oxygen itself.

⁵fever- and pain-suppressing

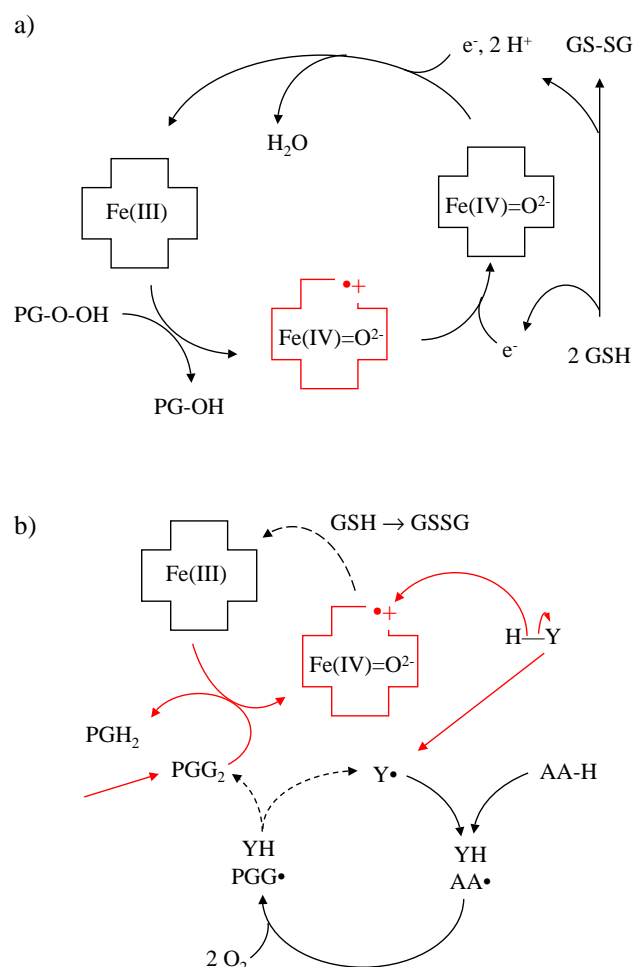


Figure 12.7. Overview of the peroxidase reaction of cyclooxygenase. a: Prostaglandin G_2 enters the peroxidase active site after leaving the cyclooxygenase site. It is reduced by heme, which thereby is converted into the cation radical form (red). The heme is reduced in turn by glutathione (GSH) in a stepwise fashion. b: ‘Priming’ of the cyclooxygenase site. The heme cation radical abstracts a hydrogen atom from Tyr 385, which thereby replaces one glutathione. The prostaglandin G (PGG) that is required to form the heme cation radical in the first place must be provided by another enzyme molecule. (AA-H: arachidonic acid)

2. Crystal structures of inhibitors bound to Cox-1 and Cox-2 have been obtained, and they can be used together with mutagenesis experiments to understand the molecular interactions of inhibitor molecules with the active site. Such knowledge is useful, since it allows the development of more selective or more effective inhibitors to proceed in a targeted way.

As an example, the binding of diclofenac to the active site of Cox-2 is shown; its carboxyl group binds to the serine 530 residue (Figure 12.8a). In contrast, the carboxyl group of indomethacin points to arginine 120 and Tyr 355, much in the same way as the carboxylate of arachidonic acid does (not shown). Results from mutagenesis experiments correspond well with these findings (Figure 12.8b): While re-

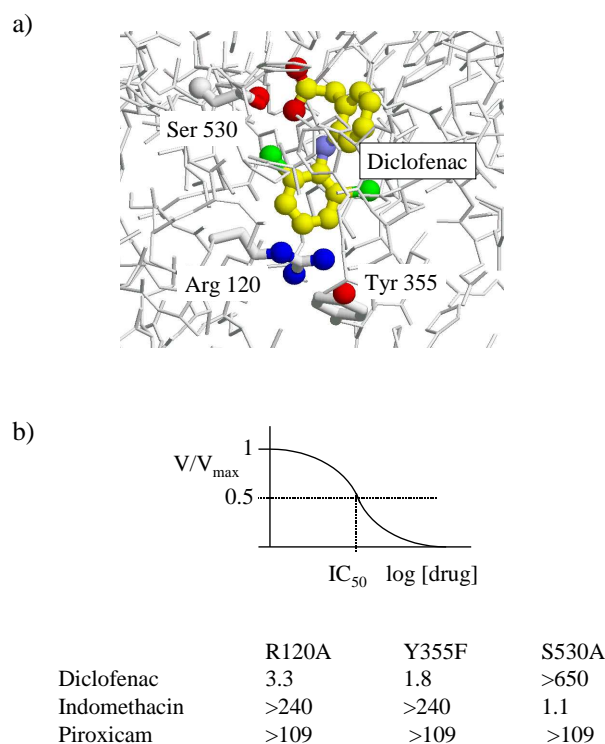


Figure 12.8. Interaction of the inhibitors diclofenac and indomethacin with cyclooxygenase 2. a: Crystal structure of diclofenac bound to the cyclooxygenase active site of Cox-2. The carboxyl group of the inhibitor is hydrogen-bonded to Ser 530. Indomethacin (not shown) adopts a different orientation, so that its carboxyl group associates with Arg 120 and Tyr 355. b: Effects of mutagenesis on the IC_{50} values of diclofenac and indomethacin as well as a third inhibitor (piroxicam). Larger IC_{50} values indicate a weaker inhibitory effect. Data from *JBC* 278, 45763-45769 (2003)

placement of arginine 120 or tyrosine 355 strongly reduces the inhibitory potency of indomethacin but not diclofenac, the opposite behaviour is observed with the removal of serine 530. All three residues are important with a third inhibitor (piroxicam; Figure 12.9).

While diclofenac and most other cyclooxygenase inhibitors act competitively (i.e., non-covalently), acetylsalicylic acid causes covalent modification of serine 530. Its effect may therefore last longer than that of a non-covalent inhibitor. Interestingly, the half-life of acetylsalicylic acid is rather short – about 15 minutes; most of the drug is just hydrolysed to acetic acid and salicylic acid. However, salicylic acid itself still acts as a (competitive) inhibitor of Cox. Also, the covalent modification of Cox achieved early on will persist after elimination of acetylsalicylic acid, so that the clinical effect of this drug will outlast its elimination.

The covalent, irreversible mode of action of acetylsalicylic acid is important in its use for inhibiting thrombocyte aggregation in patients with cardiovascular disease (more specifically, atherosclerosis). This is a practically very im-

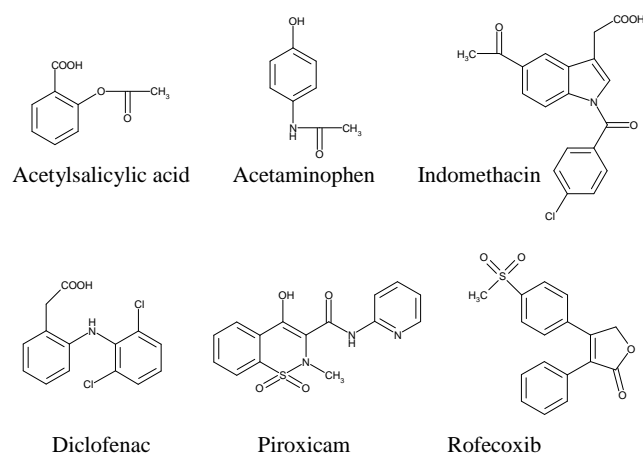


Figure 12.9. Structures of selected cyclooxygenase inhibitors. Rofecoxib is selective for Cox-2; the selectivity of acetaminophen *in vivo* is not entirely clear yet. The residual inhibitors act on both Cox-1 and Cox-2.

portant application, since atherosclerosis is a very common disease, particularly in western countries.

Thrombocyte aggregation is promoted by thromboxanes, which are synthesized in thrombocytes, and is inhibited by prostaglandins I and E, which are released by endothelial cells (Figure 12.10a). As all of these are derived via Cox-1, we need to selectively inhibit Cox 1 in thrombocytes but not endothelial cells. How can such selectivity be possibly achieved? The solution to this dilemma lies in the different lifetimes of Cox-1 in the two cell types: In endothelial cells, the enzyme is turned over within hours; inactivated enzyme molecules will thus be replaced by newly synthesized ones. Thrombocytes, however, don't have a nucleus and therefore lack protein synthesis; irreversibly inactivated enzyme molecules will therefore never be replaced (Figure 12.10b). If we properly adjust the dosage of acetylsalicylic acid, we can indeed maintain the enzyme activity in the endothelium yet efficiently inhibit it in the thrombocytes. Increasing the dosage, indeed, will reduce the beneficial drug effect, since both PGI/PGE and TXA synthesis will now be inhibited.

Another, more recent principle of limiting thrombocyte aggregation consists in the use of thromboxane receptor blockers. Examples are shown in Figure 12.11a. There even are drugs (presently experimental) that potentially inhibit both the thromboxane receptor and the enzyme thromboxane A synthase. It is rather intriguing that one such drug molecule (BM-573, Figure 12.11b) could be obtained by only slight modification of a precursor (torasemide) that has only weak inhibitory activity at the receptor, and lacks any obvious similarity with thromboxane altogether.

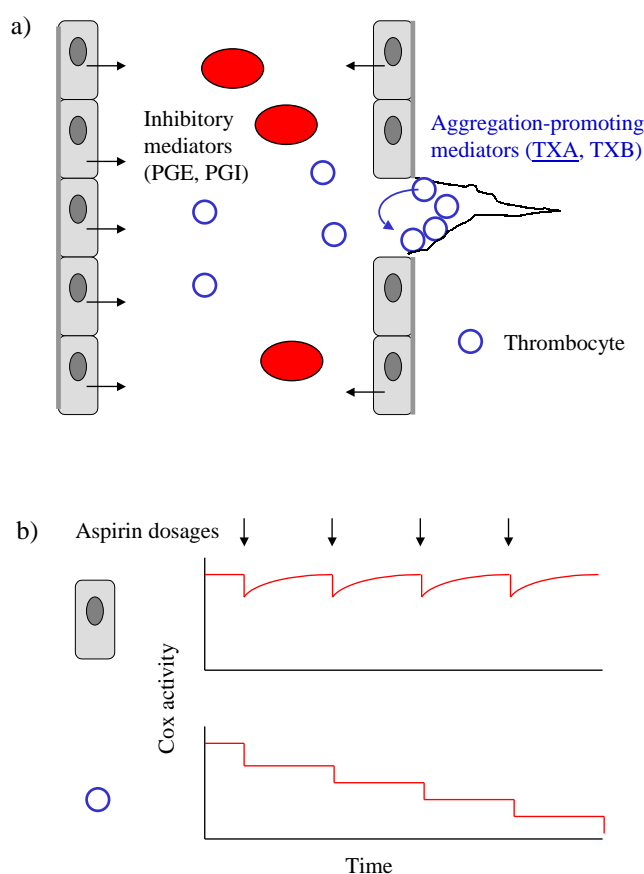


Figure 12.10. Role of eicosanoids in thrombocyte (platelet) aggregation, and rationale of low-dose acetylsalicylic acid treatment. a: Thrombocyte aggregation is suppressed by the intact vascular endothelium by a constant secretion of PGE and PGI. b: Inhibition subsides at lesions. This sets off aggregation, which is sustained and amplified by the secretion of thromboxanes by the platelets themselves. Aggregation will also promote plasmatric coagulation (i.e., fibrin clot formation). b: Effects of low dose application of acetylsalicylic acid. Endothelial cells are nucleated; covalently inactivated cyclooxygenase molecules will be replaced by newly synthesized ones, so that the activity is not substantially diminished. In contrast, thrombocytes lack protein synthesis, so that the effect of repeated doses will be cumulative.

12.3. Lipoxygenases and related drugs

The other major group of enzymes that are involved in the synthesis of eicosanoid mediators are the lipoxygenases. There are three different such enzyme specificities – 5-, 12-, and 15-lipoxygenase, which introduce a hydroperoxy group at the respective carbons of arachidonic acid. Reduction of these hydroperoxides (HPETEs) leads to hydroxyl derivatives (HETEs). These apparently are mediators in their own rights, but they can also be further converted to lipoxins by the successive action of different lipoxygenases, as illustrated in Figure 12.12b for 15- and 5-lipoxygenase. Lipoxins seem to have anti-inflammatory activity.

While the reactive groups in the active sites of cyclooxygenases and lipoxygenase are quite different, their catalytic

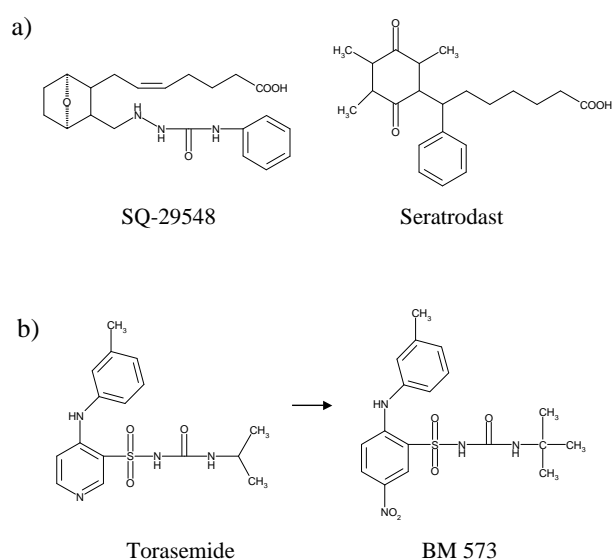


Figure 12.11. Structures of two thromboxane A receptor blockers (a), and of BM 573, a combined antagonist of both TXA receptor and TXA synthase (b). BM 573 was derived from the diuretic torasemide.

mechanisms are actually similar in that both start out with the abstraction of a hydrogen from the fatty acid and the subsequent combination with an oxygen radical (cf. Figures 12.6 and 12.12). Interestingly, while Cox-1 is completely inactivated by acetylsalicylic acid, acetylated Cox-2 is still able to catalyze the above initial steps and thus function as a lipoxygenase, giving rise to the formation of stereoisomers of physiological HETEs and lipoxins. It has been proposed that these may contribute to the therapeutic effect of acetylsalicylic acid.

For 5-HPETE, however, there is another important route, which leads to leukotriene A_4 and from there to either leukotriene B_4 or, by reaction with glutathione and subsequent cleavage of peptide bonds, to leukotrienes C_4 , D_4 and E_4 (Figure 12.13). Leukotrienes (LTD $_4$, in particular) are potent mediators in asthma, arthritis and other chronic allergies and inflammatory diseases. Inhibitors of both lipoxygenase 5 and of leukotriene receptors are available and being successfully used in clinical therapy (Figure 12.14).

Since lipoxygenases and cyclooxygenases share the same pool of arachidonic acid, one might expect an undesirable increase in the synthesis of leukotrienes upon inhibition of cyclooxygenase (Figure 12.15a). Indeed, it has long been known that patients suffering from asthma may experience clinical deterioration upon use of acetylsalicylic acid, which is in line with a prominent role of leukotrienes as mediators in this disease. Selective inhibition of Cox-2 should be less freighted with this type of interaction. Moreover, there are inhibitors (still somewhat experimental at present; Figure 12.15b) that inhibit both 5-Lox and Cox-2 but not Cox-1 – a remarkable feat of drug development, given that

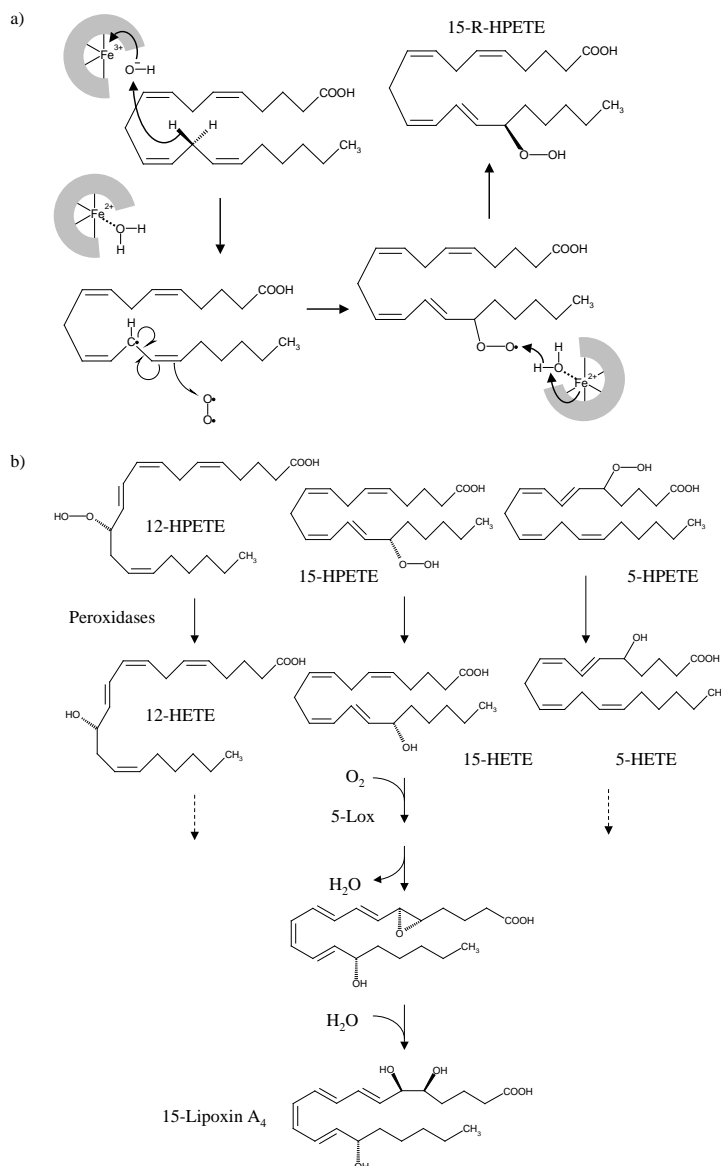


Figure 12.12. Eicosanoids derived by lipoxygenases. a: Mechanism of reaction, illustrated for 15-lipoxygenase. Abstraction of a hydrogen atom by a non-heme iron in the active site leads to a carbon radical intermediate, which subsequently reacts with an oxygen π radical. b: Products of the three lipoxygenases (5-, 12-, and 15-lipoxygenase). The initial hydroperoxy products (HPETE = hydroperoxyeicosatetraenoic acid) are reduced by glutathione peroxidases to hydroxy derivatives (HETE). HETEs may have signalling roles of their own, and they may also be converted further to lipoxins. This conversion is initiated by another lipoxygenase, as shown here for 5-lipoxygenase (5-Lox) acting on 15-HETE. 5-HPETE is also the precursor of the leukotrienes (cf. Figure 12.13).

Cox-1 and Cox-2 are more closely homologous to each other than to lipoxygenase. The complementary effect – increased formation of prostaglandin E and thromboxanes upon application of lipoxygenase inhibitors – has been substantiated by laboratory findings, but I am not aware of evidence that this has a clinical impact⁶.

Aside from sophisticated drug design, there is a surprisingly simple means of favourably influencing the balance of Good and Evil in eicosanoid metabolism: Eating fish. As initially mentioned, aside from arachidonic acid, other multiply non-saturated fatty acids can serve as precursors of eicosanoid mediators as well, in particular eicosapentanoic acid (EPA), which has an additional double bond between C17 and C18. The introduction of this ‘omega-3’ double bond⁷ does not occur, nor is it reversible, in mammalian

⁶It should be noted that this type of interaction would not be expected when using receptor blockers. Receptor blockers are often ‘cleaner’ than enzyme inhibitors.

⁷Omega (ω) is the last letter in the greek alphabet; an omega-pre-

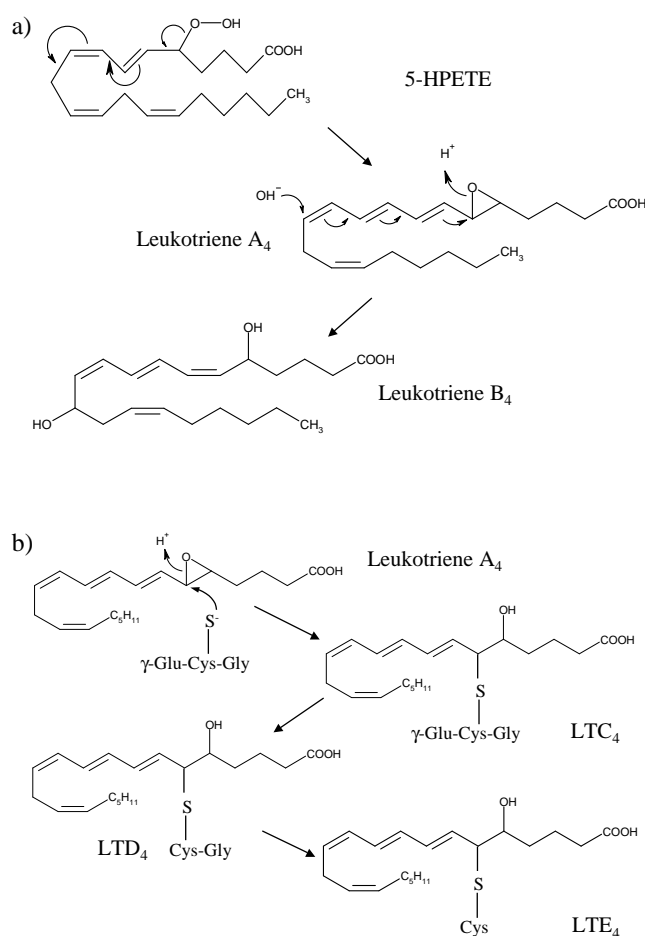


Figure 12.13. Synthesis of leukotrienes. a: Formation of leukotriene A and B from 5-hydroperoxyeicosatetraenoic acid, the product of 5-lipoxygenase. b: Successive formation of leukotrienes C, D, and E from leukotriene A. All steps are enzymatically catalyzed.

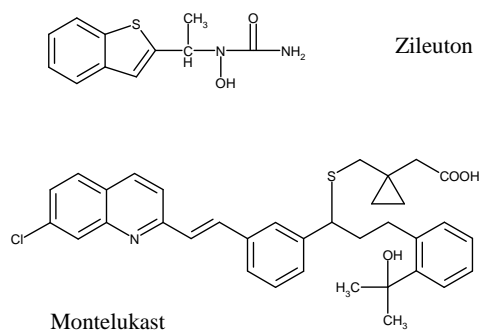


Figure 12.14. Structures of Zileuton, an inhibitor of 5-lipoxygenase, and of montelukast, an leukotriene D₄ receptor antagonist.

metabolism (Figure 12.16a). It does, however, occur in plants, where it contributes to the adjustment of membrane fluidity in cold environments. Accordingly, we find high concentrations of EPA in algae from cold water, and from

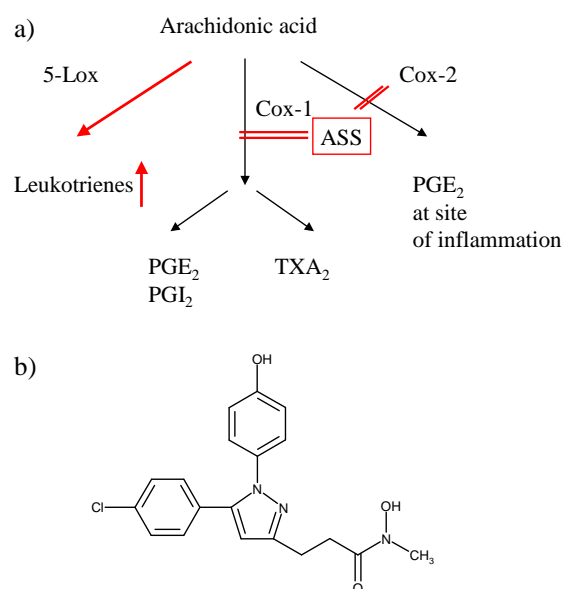


Figure 12.15. Increased leukotriene formation as an indirect consequence of cyclooxygenase inhibition by, e.g., acetylsalicylic acid (ASS; a), and structure of the 5-Lox/Cox-2 dual inhibitor tepoxalin (b).

the animals (fish) feeding on them. A high intake of this type of food will lead to a partial replacement of arachidonic acid in our own cell membranes by EPA, and therefore to an increased fraction of EPA derivatives in our eicosanoids (Figure 12.16b). Some of the EPA derived mediators are as active as those derived from arachidonic acid. However, it appears that this does not apply to thromboxane (TXA₃) and leukotrienes (LTD₅ and similar). Since these are the major culprits among the eicosanoids in sustaining inflammation and triggering thrombocyte aggregation, the result of a diet rich in fish and seaweed should be a reduction in inflammation and thrombocyte aggregation, a longer life, grumpy heirs, and impoverished physicians.

Now, does this work? There are some intriguing statistics to support this: While in western countries 40% of all deaths are due to atherosclerosis (in which both inflammation and thrombocyte aggregation are important), this number stands at 7% among Greenland eskimos and at 12% in Japan; both countries have more fish and less meat in their diets. However, it should go without saying that we cannot precisely gauge the contribution of EPA and related fatty acids to this remarkable statistics.

fixed number specifies a bond position relative to the end of the fatty acyl chain.

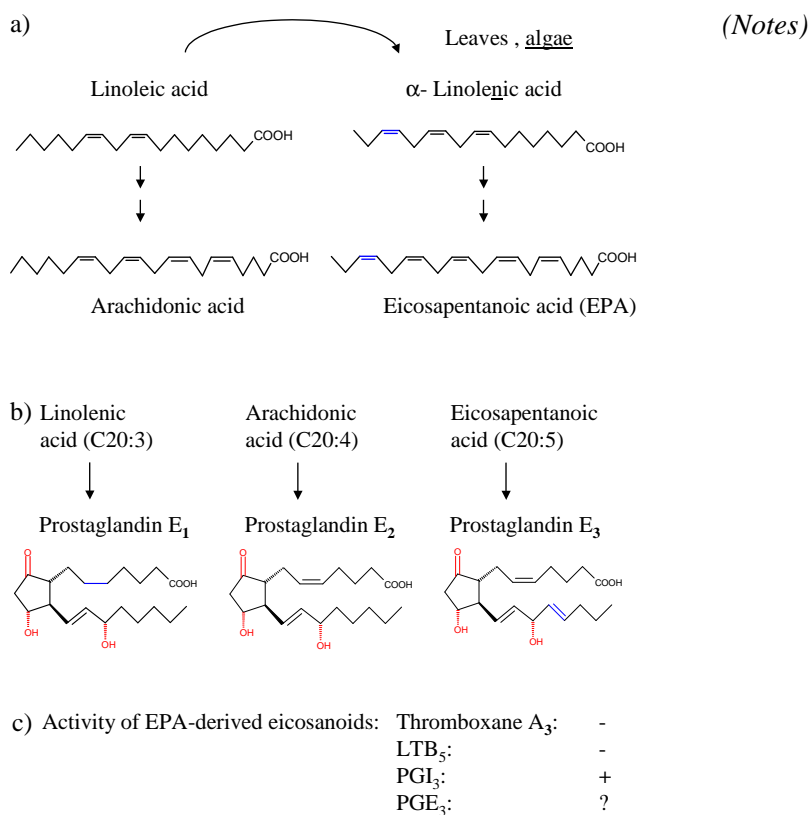


Figure 12.16. Omega-3 fatty acids and their relevance to prostaglandin metabolism. a: Omega-3 unsaturated fatty acids are derived from linoleic acid by a special desaturase found in plants but not in mammals. They are particularly common in cold-water fish. b: The different numbers and positions of double bonds persist in the eicosanoids derived from different fatty acid precursors, giving rise to different homologous derivatives. This is shown here for prostaglandin E. c: Biological activity of the eicosanoids derived from eicosapentanoic acid (EPA).

Chapter 13. Some principles of cancer pharmacotherapy

Cancer therapy consists of surgery, irradiation, and chemotherapy. Cancer chemotherapy is particular and particularly challenging in several aspects:

- While in most cases the aim of pharmacotherapy is to modulate cell function, in cancer therapy it is to kill the diseased cells – no prisoners. Selective killing is quite feasible with bacteria, since they are prokaryotic, and their biochemical apparatus contains a substantial number of targets that do not occur in human cells¹. It is more challenging yet possible with fungi and parasites. The latter share with our own cells the eukaryotic nature, and so inhibitors of ribosomal protein synthesis, which are very important in antibacterial chemotherapy, are mostly useless. However, fungi have ergosterol in their cell membranes instead of cholesterol, and both ergosterol and its synthesis are targeted by drugs that thus can be reasonably selective². In contrast, since tumour cells are derived from normal body cells, they do not normally possess any particular drug target that sets them apart from the healthy cells.
- Cancers are diverse – they may be derived from nearly any organ, and there are several types of cancers for each organ. This diversity brings with it an inherent variability in the susceptibility to chemotherapy. Some cancers are susceptible to many cytotoxic agents and can be cured quite efficiently; an example is lymphatic leukemia, especially in childhood that nowadays has a cure rate of approximately 90%. Others – e.g., squamous lung cancer – are virtually intractable if not by surgery. In many cases, the susceptibility cannot be predicted accurately beforehand. However, with an increasing number of tumours (particularly leukemias), genetic analysis is being used for exact typing, which helps in the selection of appropriate treatment regimens.
- Cancers, even if typically derived from one individual transformed cell, are heterogeneous – due to their inherent genetic instability. Normal body cells are ‘good citizens’ and obey the commandments enshrined in the genome; the same set of enzymes and receptors (i.e.,

¹ E.g., cell wall synthesis. Penicillin, vancomycin and several more antibiotics are very well tolerated simply because human cells don’t have a cell wall.

² Amphotericin B preferentially damages ergosterol-containing membranes; fluconazole and several congeners are inhibitors of ergosterol synthesis.

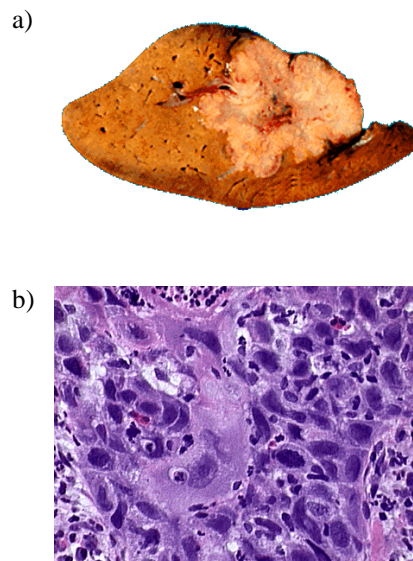


Figure 13.1. Macroscopic and microscopic appearance of solid tumours. a: A metastasis of an intestinal tumour in the liver. The tumour is the bright blob in the center, showing irregular tissue organization, and yellowish fat deposits and cavities resulting from cell degeneration. The tumour grows invasively into the surrounding liver tissue. b: Microscopic picture of a malignant epithelial (squamous cell) tumour. Note the very irregular size and shape of nuclei and of cells.

drug targets), with the same regulation and expression levels, can be expected to occur in any cell of a given type. In contrast, tumours at advanced stages usually develop multiple clones with varying chromosomal deletions, duplications and translocations that can profoundly change the prevalence of drug targets and the function of regulatory mechanisms. E.g., breast tumours that initially are susceptible to hormone withdrawal (or competitive hormone antagonists) frequently give rise to clones that grow without the hormones; tumours that initially are inhibited by anti-metabolites may develop resistance by changing expression levels of the enzymes involved.

Although we are concerned here with chemotherapy only, it is worth noting that irradiation therapy also has a chemical mode of action (Figure 13.2): Energy-rich particles (photons in X- and γ -irradiation, electrons or neutrons in particle irradiation) dispose of their energy in multiple successive events, giving rise to multiple radicals (mostly derived from water) along their path. These, in turn, may di-

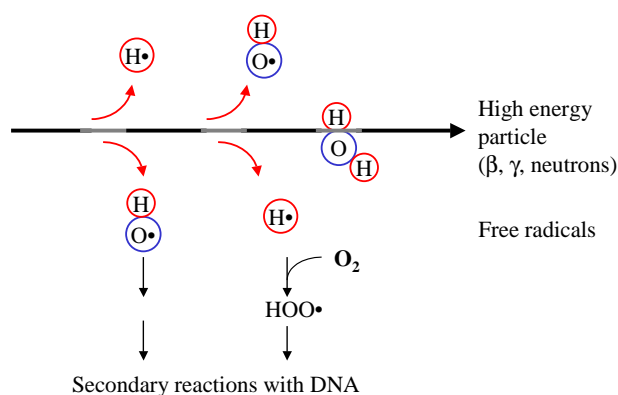


Figure 13.2. Mode of action of irradiation therapy. Energy-rich particles cleave water into radicals, which either directly or after combining with oxygen react with DNA to give rise to mutations.

rectly react with macromolecules, or they may react with oxygen to form superoxide radicals, which have a longer lifetime and thus are more likely to find biologically significant targets (mostly DNA) by diffusion. Oxygen therefore sensitizes cells to radiation, while radical-capturing and reducing compounds (e.g. free thiols) will reduce sensitivity. Radio-sensitizing and -protecting drugs for use in conjunction with radiation have been investigated but not found widespread clinical application³.

13.1. Cell type-specific antitumor drugs

Among the drugs used in cancer chemotherapy, we may broadly distinguish two functional groups:

1. Cell-type specific drugs, and
2. General cytotoxic drugs.

The availability of cell type-specific drugs is limited to selected tumours and depends on some specific traits the tumour cells have inherited from their differentiated, healthy ancestors. Most commonly, this is the dependence of proliferation on a particular hormone, or the the inhibition of growth by a mediator. Examples:

1. Many breast cancers, like normal breast gland cells, are dependent on estrogens and / or gestagens for their growth. Receptor antagonists for estrogens (e.g., tamoxifen) or gestagens (mifepristone, Figure 13.3) will therefore stop or delay growth of these cells. Of course, these drugs will affect other hormone-dependent tissues as well – e.g., mifepristone will also disrupt the function of the placenta in pregnancy, and in fact is used more commonly for abortion than for cancer treatment.

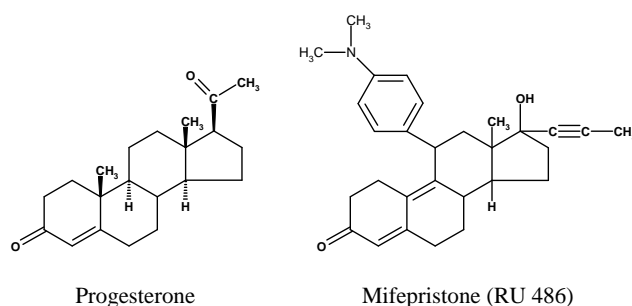


Figure 13.3. Structures of the gestagene steroid hormone progesterone, and the progesterone receptor antagonist mifepristone

2. Hairy-cell leukemia is a rare form of lymphatic⁴ leukemia. Proliferation of the malignant cells is very efficiently inhibited by interferon- α . This is one of the few cases so far in which a ‘biological’ therapy has actually lived up to its promise.
3. Promyelocytic leukemia is caused by a very specific genetic event – the reciprocal translocation of some segments between chromosomes 15 and 17 (Figure 13.4). The ‘rift’ of translocation goes right through the gene for α -retinoic acid receptor, which therefore is expressed in two deviant forms. Retinoic acid, via its receptors, acts as a transcriptional regulator in the final differentiation of the promyelocytes to granulocytes. This differentiation is arrested by the functionally deviant receptors. Promyelocytes proliferate, while granulocytes don’t; arrest of differentiation therefore leads to the build-up of a proliferating yet useless (even harmful) cell population. However, it is possible to restore differentiation to the aberrant cells by the application of retinoic acid in larger than physiological amounts. This is probably due to the fact that the cells are diploid, and the second set of chromosomes 15 and 17 is intact, thus providing for an intact copy of the α -retinoic receptor gene⁵.
4. Another example of cell type-specific chemotherapy is the use of mitotane (Figure 13.5) in adrenal gland cancer (those derived from the cortex, not the medulla of the adrenal gland). Selective toxicity is conferred by the enzyme 11- β -hydroxylase, a member of the cytochrome P450 family that is located in the mitochondria of the adrenal gland cortical cells and participates in the synthesis of glucocorticoids (e.g., cortisone). Mitotane is a substrate⁶ for this enzyme, which converts it to an acyl

³It is not very useful to simply increase or decrease toxicity; the challenge is to improve selective toxicity for cancer cells.

⁴Leukemias are classified according to the lines of blood cell precursors they arise from. Lymphatic leukemia and myeloid leukemia are most common.

⁵This is actually an exception to the rule that cancer chemotherapy is tantamount to cell killing.

⁶There are others, including environmental degradation products of the infamous insecticide DDT.

chloride. The acyl chloride in turn reacts with several proteins in the mitochondria, which will irreversibly damage the cells.

Importantly, with all of the above cell-specific therapies, resistance is common, either primarily (no response to begin with) or secondarily (initial response but emergence of non-responsive cell clones). These therapies therefore

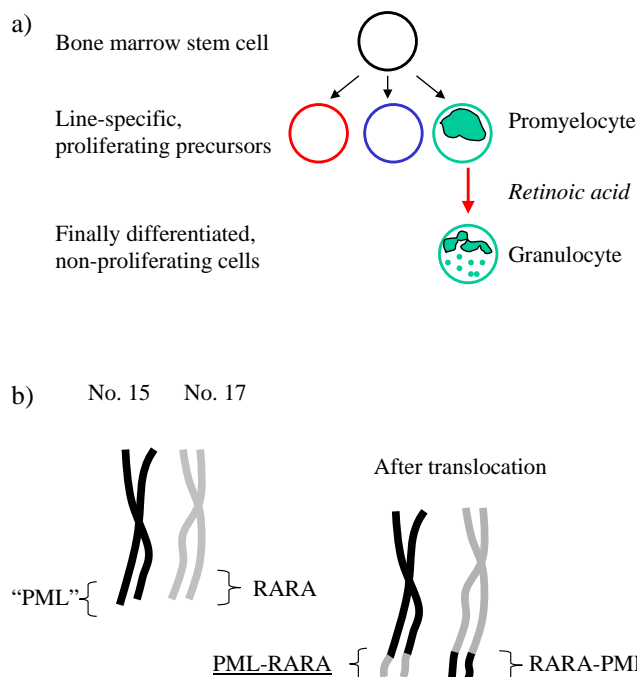


Figure 13.4. Promyelocyte leukemia, and its relationship to α -retinoic acid. a: Promyelocytes are an intermediate stage in granulocyte differentiation. They are able to proliferate. Their final differentiation into granulocytes (which do not divide anymore) depends on the transcriptional effects of retinoic acid. b: In promyelocytic leukemia, the gene for the retinoic acid receptor is disrupted by a chromosomal translocation, so that maturation stalls, and promyelocytes continue proliferating.

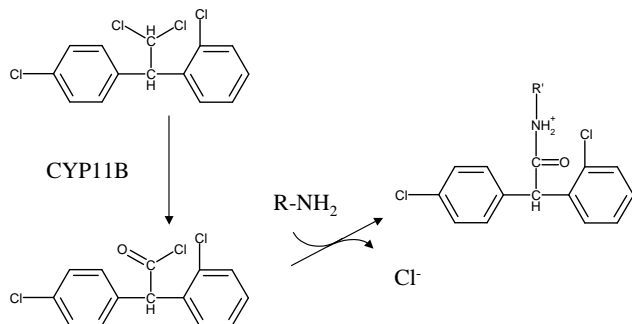


Figure 13.5. Structure of mitotane, its acyl chloride conversion product formed by the enzyme cytochrome P450 11B (CYP11B), and the reaction products of the latter with amino groups in proteins or nucleic acids. CYP11B occurs in the mitochondria within the adrenal cortex, rendering this tissue (and its derived tissues) selectively susceptible to mitotane.

typically have to be combined or followed up with one of the general, non-cell-specific cytotoxic therapies described below.

13.2. The cell cycle

General cytotoxic drugs may be classified according to their relationship to the cell cycle (Figure 13.6). Cell cycle-specific agents may affect either the S phase (i.e., DNA synthesis) or the M phase (i.e., mitosis). Cell cycle-nonselective agents may damage the cell throughout the cycle, mostly by direct chemical modification of the DNA.

13.3. Alkylating agents

Synthetic drugs that are not cell cycle-specific are mostly alkylating agents. They have diverse reactive groups. Several drugs share the ‘N-mustard’ structure shown in Figure 13.7a. The reaction mechanism (formation of aziridine intermediates that react as electrophiles; Figure 13.7b) is actually the same as previously discussed for the irreversible α -blocker phenoxybenzamine; here, however, we don’t have a moiety that targets the drug to any particular protein. It should go without saying that most molecules will actually not react with DNA but instead with some other nucleophile hopping about in the cell, in particular glutathione or other sulfhydryls. However, those that do react with DNA are the ones that matter, since the harm done by them has the potential to be permanent. Also note that we have not one but two chloroethyl groups – this creates the possibility of introducing cross-links into the DNA⁷. Cross-links between the two strands of the DNA bases are more likely to give rise to permanent mutations than modifications affecting one strand only, since they cannot be removed by exci-

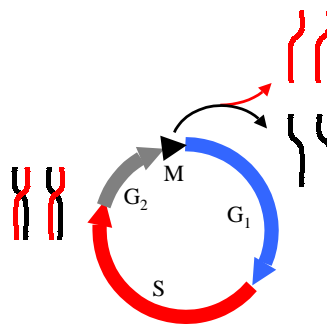


Figure 13.6. The cell cycle. The M phase (mitosis) and the S phase (DNA synthesis) are targeted by different types of cell cycle-specific cytotoxic drugs.

⁷Or of cross-linking the DNA with some other molecule, e.g. a protein, which might be even more deleterious (I don’t know whether this has actually been studied).

sion repair (although there are ‘error-prone repair’ mechanisms that may remove them).

The most common target of alkylation – quite independently of the alkylating agent used – is the N7 in guanine (Figure 13.7b,c). Why is that? The six-membered ring that is part of the guanine is locked up in base pairing within the double helix, i.e. inaccessible; this lets out the other nitrogens. The same applies to the other nucleotides. Why would N7 in guanine be more commonly affected than N7 in adenine? The guanine ring is not as completely aromatic as the adenine ring is. The π electrons of the ring nitrogens are therefore not as completely delocalized, i.e. the nitrogens will be stronger nucleophiles. However, the pref-

erence is not absolute, and alkylations of adenine and the pyrimidine bases do occur as well.

An interesting consequence of guanine N7-alkylation is the increased propensity of the guanine ring to adopt the tautomeric form (Figure 13.7c). In this form, the arrangement of hydrogen bond donors and acceptors is reversed and now resembles that of adenine, thus enabling guanine to base pair with thymine instead of cytosine. This is believed to contribute to the mutagenic effect of guanine alkylation.

One very commonly used agent containing the dichloroethyl-amine moiety is cyclophosphamide. This drug may actually be metabolized quite extensively and give rise to several toxic metabolites, the exact contribution of which to the overall therapeutic effect is not very well established (Figure 13.8a). Metabolism is initiated by a cytochrome P450 enzyme in the liver (and possibly elsewhere) and continued by several other enzymatic and non-enzymatic steps. One of the final decay products is acrolein⁸, which may form several adducts with guanine, some of which are shown in Figure 13.8b. While the metabolites of cyclophosphamide such as acrolein and chloroacetaldehyde seem to be quantitatively more important in the ultimate reactions with DNA than the parent drug itself, the relative significance of individual metabolites is somewhat hard to determine from the literature.

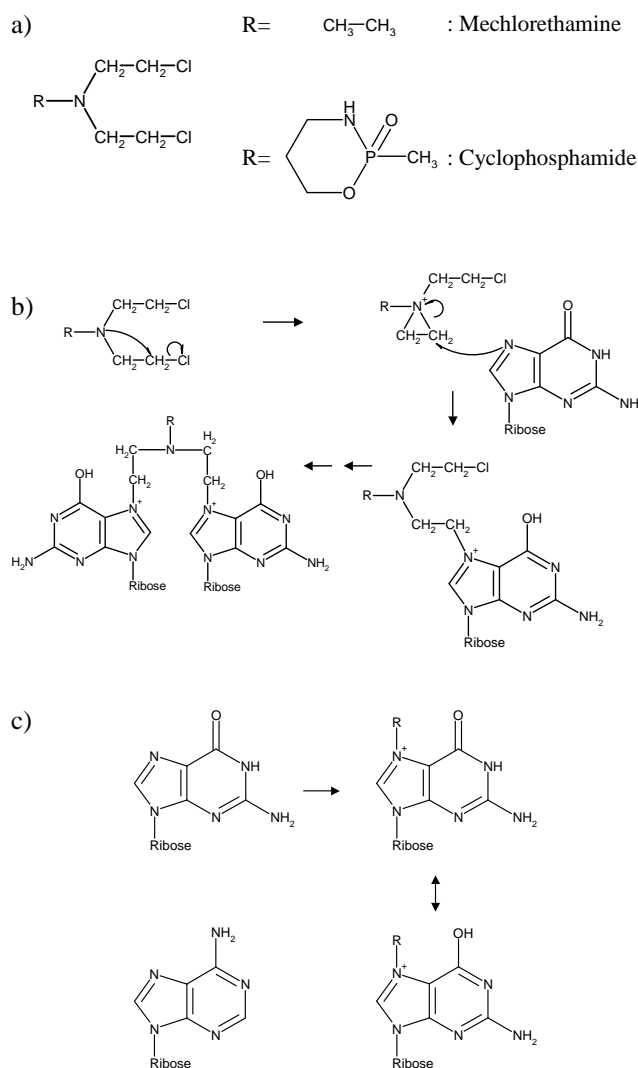


Figure 13.7. Some alkylating agents and their reactions with DNA. a: The ‘N-mustard’ (di-[chloroethyl]-amine) group is found in several agents such as mechlorethamine and cyclophosphamide. b: Successive reaction of the N-mustard group with two guanine bases, potentially forming a cross-link between the two strands of a DNA molecule. c: Tautomerisation of guanine, after alkylation of its N7 position. Adenosine (bottom left) is shown for comparison.

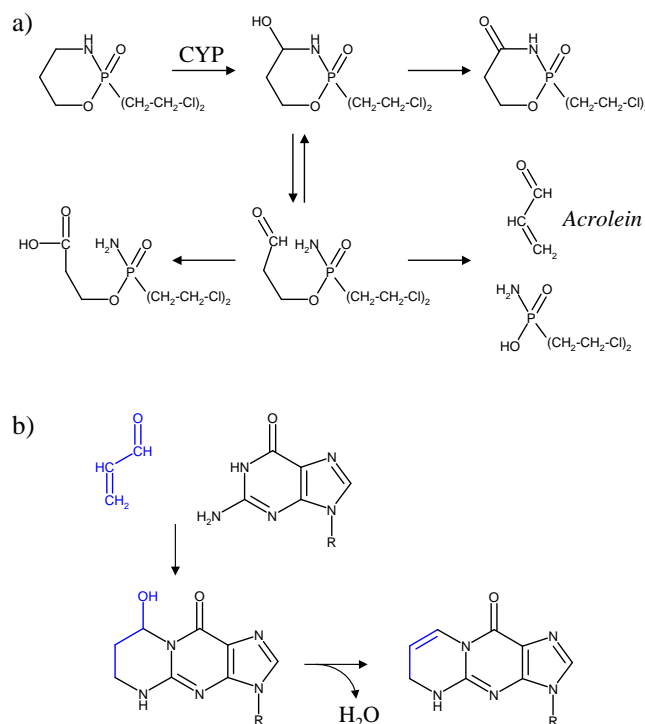


Figure 13.8. Metabolism of cyclophosphamide (a), and reaction of one major metabolite, acrolein, with guanine (b).

⁸Acrolein also forms when frying food and may be one of the agents responsible for the rising frequency of colon cancer in western countries.

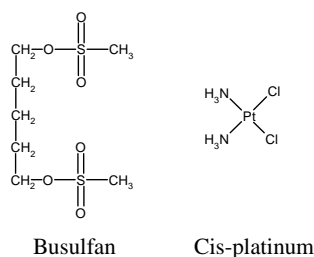


Figure 13.9. Structures of two alkylating agents that are commonly used in tumour therapy.

Alkylating agents with other reactive groups do exist – e.g., busulfan and cis-platinum (Figure 13.9) – but we will lightly gloss over their respective intricacies, in particular the chemical mechanism of cisplatin and only note that the preferentially formed lesion observed with the latter drug again involves guanines; two that are located adjacently within the same strand become cross-linked to each other.

What are the biological consequences of the covalent modifications caused by alkylating agents? One that we have already noted is the introduction of mutations opposite a modified base. Another one is the inhibition of DNA synthesis; e.g., some of the acrolein adducts of guanine (Figure 13.8b) inhibit the incorporation of any base opposite to them, thus interfering with DNA synthesis and repair. Of note, many of these chemical adducts are removed by DNA repair enzymes only inefficiently or not at all; the enzymes are apparently not ‘accustomed’ to these peculiar types of modifications⁹.

13.4. Antibiotics

Another class of DNA-damaging drugs are found in nature as antibiotics. Antibiotics are widely used by competing soil microorganisms, both prokaryotic and eukaryotic. While many of them are selectively toxic for prokaryotic cells and therefore of use in antibacterial therapy, an even larger number is toxic for both prokaryotes and eukaryotes, often with higher toxicity for the latter. Several of these antibiotics have been useful in biochemical research because they can be used to dissect various stages of gene expression and protein maturation.

The antibiotics used in cancer chemotherapy are mostly DNA-intercalating agents. An example is provided by daunorubicin (Figure 13.10). This drug molecule has a large, flat polycyclic system, which will intercalate between the stacked base pairs of the DNA. However, there is more to it: The ring is also able to chelate iron, and the complex

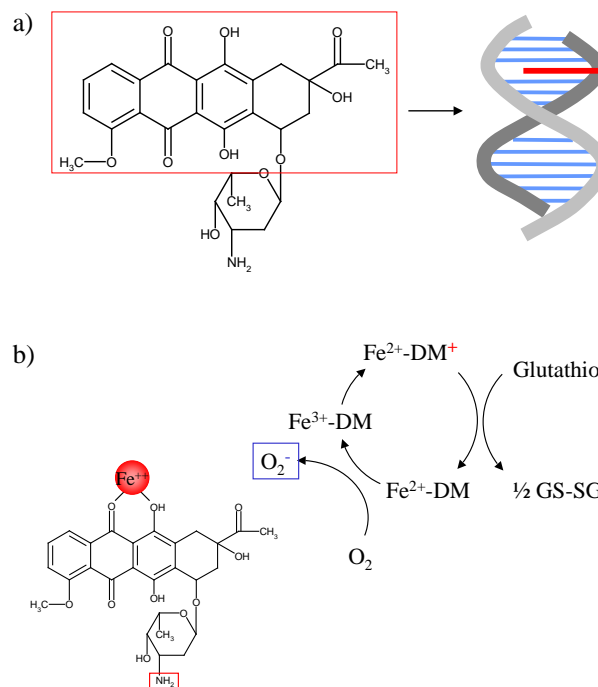


Figure 13.10. Structure of daunorubicin (daunomycin) and its intercalation into DNA (a), and catalytic formation of peroxide anions by iron complexed to it (b). Reactive oxygen species released in the immediate vicinity of the DNA will form strand breaks and also promote formation of covalent adducts of daunorubicin and DNA that involve the amino group of the former.

catalyses the formation of superoxide anions at the expense of glutathione (Figure 13.10b). The highly reactive superoxide can wreak all kinds of havoc on the DNA in its immediate vicinity, including strand breakages¹⁰. Another effect is the formation of covalent adducts between the amino group at the sugar moiety and guanine residues in the DNA. In the chapter on drug metabolism, we already saw something similar: The intercalating agent benzpyrene also required covalent reactivity for significant DNA damage to occur, which in that case was brought about by metabolic activation to the epoxide.

13.5. Antimetabolites

Cell cycle-selective agents comprise antimetabolites (of DNA synthesis) and inhibitors of mitosis (cell division; see below). A widely used example of an antimetabolite is 5-fluorouracil (5-FU; Figure 13.11). Before this drug actually does something interesting, it needs to be converted to the nucleotide analog 5-fluoro-deoxyuridinemonophosphate (5-FdUMP), which occurs in the same way as with normal uracil.

⁹On the other hand, natural radioactivity has been noticeable throughout evolution, and radiation-induced damages are usually efficient triggers of DNA repair.

¹⁰The superoxide formation is also responsible for the toxic action of daunomycin on the heart muscle.

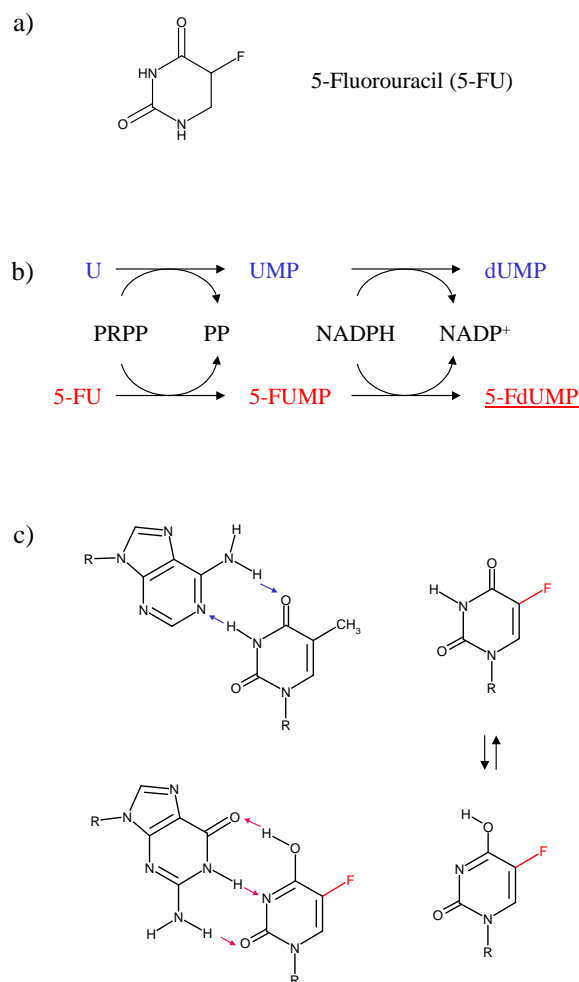


Figure 13.11. Structure of 5-fluorouracil (a), its activation by pyrimidine salvage enzymes (b), and its tautomerism that promotes ambiguous base pairing (c). While 5-FU is not very efficiently incorporated into DNA, the same mechanism applies to 5-bromouracil, which because of its closer steric resemblance of thymidine is readily incorporated.

5-FdUMP is an analog of dUMP, which is the substrate for dTMP synthesis by thymidylate synthase; it is this reaction that is inhibited by dUMP (Figure 13.12a). The catalytic mechanism of thymidylate synthase is depicted in Figure 13.12b. The enzyme (thymidylate synthase) requires N,N' -methylene-tetrahydrofolic acid as a cosubstrate. The reaction is initiated by a cysteine residue in the active site of the enzyme and involves an intermediate in which the enzyme, the substrate (dUMP), and the cosubstrate are all covalently bound (bottom center in Figure 13.12b). This complex is resolved in the second step, which involves abstraction of the hydrogen in position 5 of the uracil by a basic residue in the active site. The trick with 5-FU is that this abstraction doesn't happen, since position 5 is occupied by fluorine, which is very tightly bound to the ring. Therefore, the enzyme remains covalently locked up – 5-FU is a covalent and thus very efficient inhibitor of thymidylate synthase.

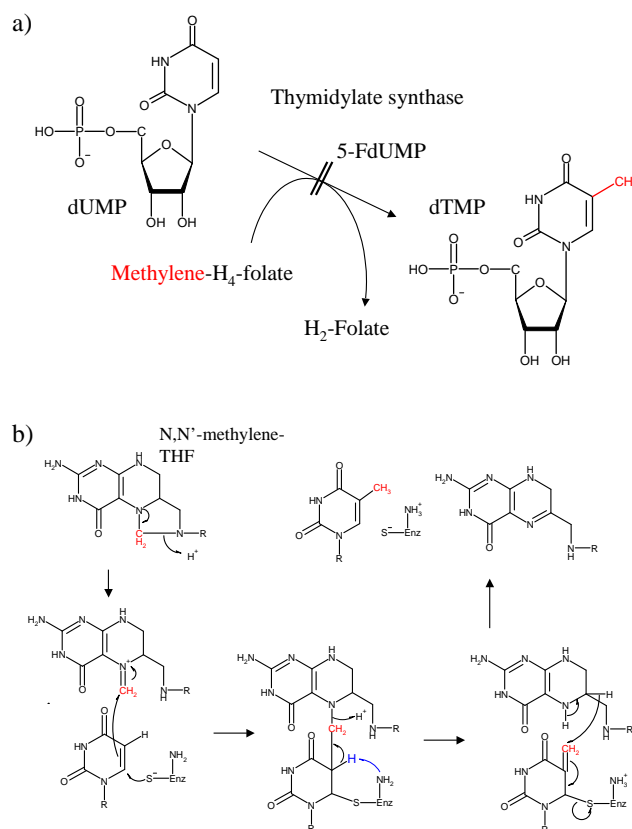


Figure 13.12. The thymidylate synthase reaction, and its inhibition by 5-fluoro-deoxyuridinemonophosphate (5-FdUMP). a: Overview of the reaction. b: The catalytic mechanism. The enzyme forms an intermediate in which it is covalently linked to both the substrate (UMP) and, via the latter, to the coenzyme (bottom center). Resolution of this intermediate does not happen with 5-FdUMP because it requires abstraction of the hydrogen normally found in position 5 of the uracil ring.

Another intriguing consequence of the fluorine substitution is its promotion of the tautomeric form of the ring, which has base-pairing properties resembling those of cytosine. Incorporation of 5-FU (subsequent to further phosphorylation of 5-FdUMP to 5-FdUTP) therefore induces mutations due to misincorporation of guanine instead of adenine (Figure 13.11c)¹¹. The same behaviour is observed with the bromine analog of 5-FU (5-bromouracil). Bromine is similar in size to a methyl group, so that 5-BU sterically resembles thymidine and therefore is efficiently incorporated into the DNA (more so than 5-FU). It is not used in cancer therapy but is commonly used (in the form of a pro-drug, 5-bromouracil-deoxyriboside) as a mutagen in experimental research.

We have just seen that folic acid functions as a coenzyme in the synthesis of dTMP. It also donates methyl groups in the synthesis of purine bases, so that it is actually quite impor-

¹¹ This is complementary to what we saw above with N7-alkylated guanine.

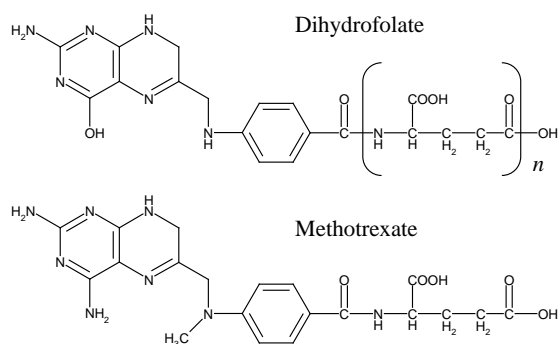


Figure 13.13. Structures of dihydrofolate and of methotrexate, a competitive inhibitor of dihydrofolate reductase.

tant in DNA synthesis. After transfer of the methyl group by N,N'-methylene-tetrahydrofolate, the remainder (dihydrofolate) is regenerated in two steps, the first of which is the reduction to tetrahydrofolate by dihydrofolate reductase. This enzyme is inhibited by methotrexate (Figure 13.13). Note that with this antimetabolite there is no possibility of introducing mutagenic base analogs into the DNA. It therefore has less carcinogenic potential than most other drugs discussed here and is also sometimes used as an immunosuppressive agent in diseases other than cancer¹².

A quite unusual antimetabolite is the enzyme L-asparaginase, isolated from *E. coli*, commonly used in the treatment of leukemia. Asparagine is a precursor of purine synthesis (Voet & Voet have all the details), and depletion of this amino acid seems to slow down tumour cells. One could speculate at length why this would have a preferential effect on tumour cells, but it may be better not. Of note, this drug can be used for extended periods of time without inducing a neutralizing immune response (which it normally should) because the immune system will be quite knocked out under the prevailing conditions of disease and treatment.

A nucleotide antimetabolite that carries the modification in the sugar rather than in the base is cytosine arabinoside (araC; Figure 13.14). In this molecule, there is an OH group in position 2 of the ribose, pointing in the 'wrong' direction (as compared to ribose). AraC gets incorporated into DNA but then apparently interferes with further DNA synthesis. This may affect different DNA polymerases to different extents; in fact, araC reportedly inhibits DNA repair more strongly than DNA replication (the two processes involve different DNA polymerases).

¹²The underlying rationale still being similar – specific immune reactions strongly depend on cell (lymphocyte) proliferation. Folic acid is a vitamin, and a lack of this vitamin gives rise to anemia. There are fewer erythrocytes in this condition, but those present have a higher than normal hemoglobin content. Synthesis of proteins is not inhibited, and the erythrocyte precursor cells accumulate more protein because they divide less rapidly.

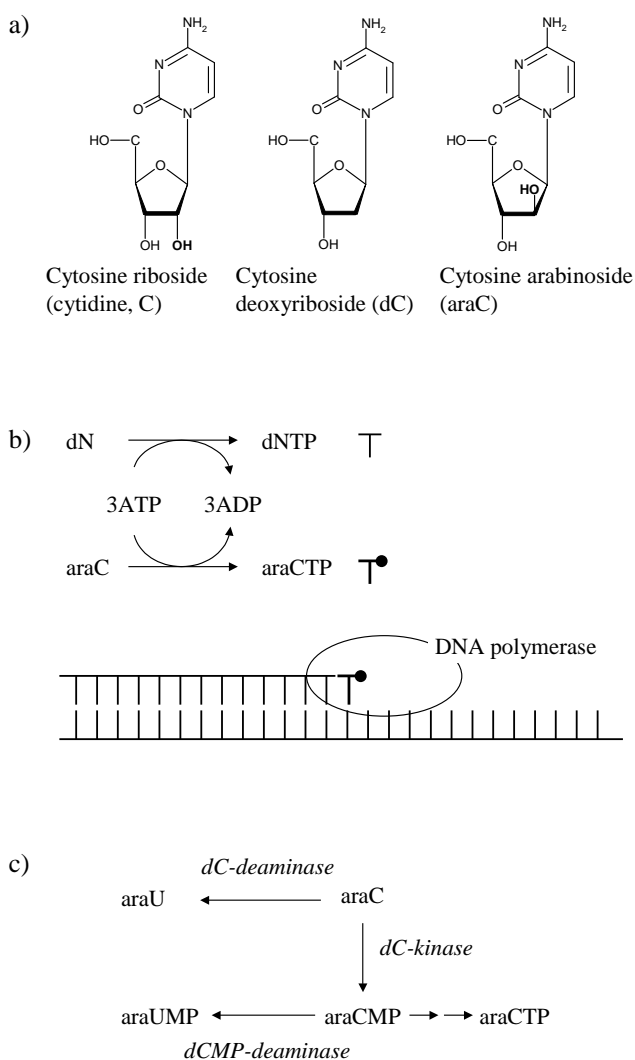


Figure 13.14. Cytosine arabinoside: Structure (a), inhibition of DNA polymerase (b), and metabolism (c).

Another aspect of antimetabolite therapy exemplified by araC is the emergence of resistance in tumours that are initially susceptible. How come? Like 5-FU and many other antimetabolites, araC requires metabolic activation; on the other hand, the activated metabolites are also subject to degradation (Figure 13.14c). In the beginning, we noticed that tumour cells are quite unstable genetically. Chromosomal deletions or duplications may easily result either in a reduced drug activation or in accelerated degradation, by way of changing the copy numbers of the genes encoding the respective enzymes. Similarly, translocation may cause transfer of these genes into foreign regulatory contexts with concomitant over- or under-expression. Such causes of cancer cell drug resistance have been experimentally confirmed.

13.6. Inhibitors of mitosis

Inhibition of mitosis is caused by drugs that interfere with the polymerization of tubulin. The polymers of tubulin

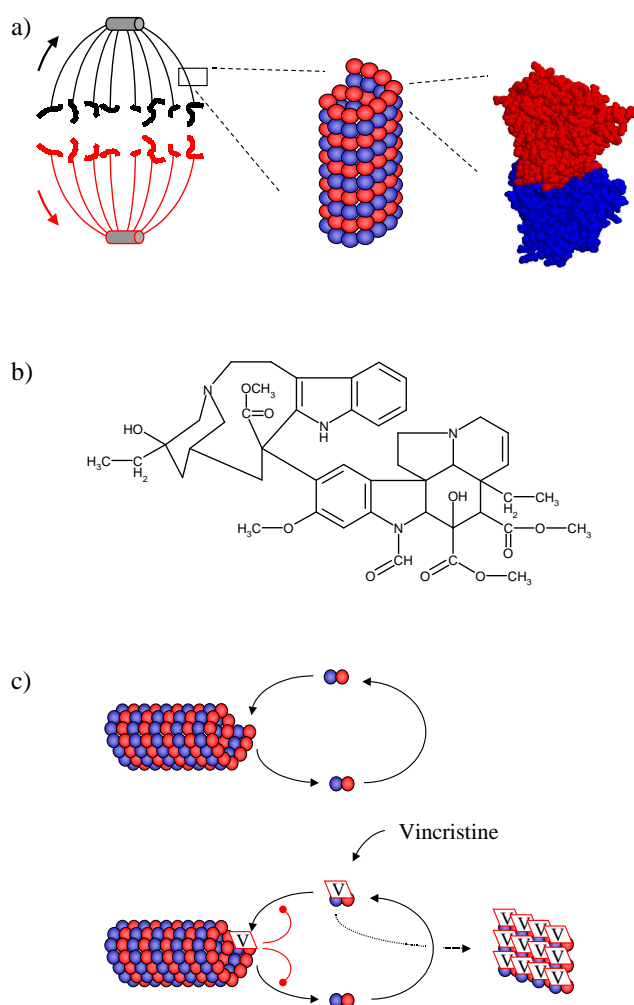


Figure 13.15. Inhibition of mitosis by vincristine. a: The mitotic spindle consists of tubulin fibers, which are helical polymers of α,β tubulin dimers. b: Structure of vincristine, an inhibitor of tubulin polymerization. c: Mode of action of vincristine. The drug binds to an α,β dimer. These dimers retain the ability to associate with growing filaments; however, the further attachment of dimers is inhibited. At high concentrations, vincristine-associated tubulin is also sequestered within mis-assembled polymers.

consist of α,β dimers, assembled in a helical fashion into a hollow fiber. These tubules are dynamically being formed and destroyed as needed by the cell; polymerization is GTP dependent. They are important in molecular transport and motion; broadly speaking, tubulin is to dynein what actin is to myosin. However, tubulin-dynein participates in other processes of molecular motion than actin/myosin, including the segregation of the chromosomes in mitosis. The mitotic spindle is the tubulin framework assembled for this purpose (Figure 13.15a). Formation of the spindle is inhibited by vincristine (Figure 13.15b). This drug molecule has a very nice complex structure, but I have not found any information on how it actually fits to the target protein (tubulin). In any event, binding of vincristine still permits the tubulin dimer to associate with the nascent tubule; howev-

er, it then inhibits the addition of further subunits. Also, it stays rather firmly in place, so that the tubule in effect becomes ‘capped’ and trapped (Figure 13.15c). Monomeric tubulin becoming saturated with vincristine will also polymerize into atypical shapes; this, however, seems to be limited to high drug concentrations and not to occur in vivo. Of note, another drug with virtually the same mechanism of action is colchicine, which however is used in the treatment of gout rather than cancer. I have not seen any rational argument for the preferment of either drug in their respective applications.

Finally, after learning about the modes of action of all these rat poisons, there remains one nagging question: Why is it that they possess any useful degree of selectivity for cancer cells as opposed to healthy body cells at all? Sure, the side effects of cytotoxic cancer therapy are considerable, most prominently (and unsurprisingly) affecting the most rapidly proliferating tissues such as bone marrow and intestinal mucous membranes. Still, in many cases conditions can be found in which the tumour suffers a much more pronounced setback than the normal tissues. Several types of tumours, including childhood leukemias and testicle tumours, are nowadays being cured by chemotherapy in the majority of cases, without severe long-term damage to the rest of the organism (remember Lance Armstrong?)¹³. While a high proliferation rate is certainly an important determinant of sensitivity to cytotoxic agents, it is worth pointing out that the mitotic rates of most tumours do not come close to the regeneration rates of the healthy bone marrow – a human being generates a whopping 600 kg of erythrocytes and leukocytes each during a lifetime!

13.7. Monoclonal antibodies in tumour therapy

While the picture given so far looks pretty bleak, there is hope of improvement. Among several new approaches to tumour therapy, monoclonal antibodies have started to make the most substantial contribution to improving its effectiveness and reducing the severity of side effects.

The use of antibodies is based on the fact that, due to altered gene expression patterns, many tumours possess one

¹³ The impressive improvement seen with some (but not all) tumours has been achieved not so much by more sophisticated principles of drug action – most drugs still work by the same principles known for decades – but by patient and carefully controlled, large-scale clinical trials with varying drug combinations, dosages, and schedules. As a general rule, combining and switching drugs is key, since this makes it so much harder for the tumours to develop resistance. The same principle is applied in the chemotherapy of chronic infections such as tuberculosis and AIDS. Also note that the mutagenic effects of the cytotoxic treatment lives on – all cured patients are at an enhanced risk of developing a second cancer (of unrelated cell type) later in life. The most conventional of all wisdoms applies – no free lunch.

or more surface proteins that are not found on the healthy cells¹⁴. Antibodies that bind to cell surface antigens can induce cell destruction in various ways:

1. By activating the complement system. This is a system of serum proteins that will bind to cell surface-bound antibodies and form holes in the cell membrane, thereby killing the cell. A straightforward example of this process is the destruction of blood cells by the transfusion of blood with incompatible blood type.
2. By activating cytotoxic lymphocytes. This process is called 'Antibody-dependent, cell-mediated cytotoxicity', often referred to by the acronym ADCC¹⁵ and is important in the immune response to virus infection.
3. Sometimes, the target protein itself will respond to the binding of antibodies with some cytotoxic effect. You have probably heard about apoptosis (programmed cell death). This is triggered by specific cell surface receptors, and some antibodies can mimic the effect of agonists at these receptors.

In addition, toxic activity can be conferred on antibodies by way of conjugating them with toxic agents (such as diphtheria toxin) or short-lived radioisotopes (such as ¹³¹I).

The first demonstration that antibodies can be of use in tumour therapy actually predates the invention of monoclonal antibodies; however, the uniformity of monoclonal antibodies makes them superior for the following reasons:

- They can be selected to be highly specific for one individual target protein. In contrast, polyclonal antibodies (even if directed against an individual target protein) have a higher potential to cross-react.
- They are standardized. In contrast, polyclonal antibodies will contain a mixture of specificities that will vary with each donor individual.
- In exceptional cases, monoclonal antibodies may be directed against a crucial epitope¹⁶ that activates a cytotoxic activity of the target protein.

For technical reasons, monoclonal antibodies initially were purely murine, i.e. derived from mouse cell lines. In clinical use, these murine antibodies met with limited success. Reasons for this include

- production of neutralizing human anti-mouse antibodies. This led to rapid elimination of the murine antibodies.

- inadequate recruitment of immunological effector functions. The interaction of murine antibodies with human lymphocytes and complement is less efficient than that of human antibodies.

A big step forward was the invention of 'humanized' monoclonal antibodies, which are hybrids in which a large part of the murine antibody molecules have been replaced by human sequences (obviously by recombinant DNA technology). In the recent years, the list of such antibodies that have received approval and are being used in practical therapy has grown to some 20 members. Not all of these are actually intended for tumour therapy; other applications include chronic inflammatory diseases such as transplant rejection, Crohn's disease or rheumatoid arthritis. Among those antibodies that are being used in cancer therapy, most are directed against target antigens that are associated with specific tumour types. For example, the first clinically useful monoclonal anti-tumour antibody (with the poetic name rituximab) is directed against CD20, a surface antigen found in B lymphocytes and particularly highly expressed in B-cell lymphomas, which are tumours derived from B lymphocytes. CD20 is a particularly suitable target antigen, for the following reasons:

- It is highly expressed on the B-cell lymphoma cells, but not on most other cells of the body.
- It remains on the cell surface after binding of the antibody, so that the antibody can trigger complement and ADCC. In contrast, many other antigens are being shed from the cells or internalized upon antibody binding, which interferes with a targeted cytotoxic action.
- CD20 has a role in apoptosis, and binding of the antibody stimulates apoptosis. This means that even without recruitment of complement or ADCC antibodies can trigger a cytotoxic effect.

It is clear that we will not always be so lucky to find such a great target on our tumour of interest. However, while B-cell lymphomas are among the less widespread (and more tractable) tumours, a more recent breakthrough is the development of an antibody that is being used in the therapy of breast cancer, which is one of the most common forms of cancer. This antibody (called trastuzumab) binds to human epidermal growth factor receptor 2 (HER2; hence trastuzumab's commercial name 'herceptin'). This receptor is overexpressed on breast cancer cells. Intriguingly, HER2 overexpression is particularly pronounced on those cells that express low amounts of steroid hormone receptors, so that trastuzumab is valuable as a complement to steroid hormone antagonists such as mifepristone. Introduction of trastuzumab has resulted in clear-cut improvements of therapy, and the only impediment to its general use is now cost¹⁷.

¹⁴Even if they are found on some healthy cell types, side effects should be more limited than with the previously discussed agents

¹⁵Not to be confused with ACDC, which are rather cytotoxic too, but selectively for the cells in the inner ear.

¹⁶Epitope: The binding site of an antibody molecule on its antigen.

As with the traditional types of tumour therapy, the success of antibody therapy is always endangered by the potential emergence of resistant clones. To reduce this risk, antibodies are typically used in combination or alternatingly with other types of agents.

(Notes)

¹⁷More than elsewhere, in the field of recombinant DNA technology, patent legislation has been abused to divert the gains accruing from public investments (into the academic sector) into private pockets, and we all are paying the price for it. I wonder why Jack Layton has not seized on this cause yet.

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